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CHARACTERIZATION OF HYBRID RICE WITH BROAD-SPECTRUM RESISTANCE TO PHILIPPINE RACES OF *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

Breeding and development of resistant cultivars has been the most effective and economical strategy to control bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Thus, evaluation of the introgressed resistance genes (*Xa4* and *Xa21*) in improved parent lines and F_1 hybrid PR40638H against the 14 most prevalent *Xoo*isolates in the Philippines, and comparison of the morpho-agronomic characteristics of the original and improved parent lines and F1 were conducted. Polymerase chain reaction (PCR) assay using sequenced tagged site (STS) markers showed that the improved parent lines and F1 hybrid contained *Xa4+Xa21*, whereas the original parent lines and F1 hybrid contained only *Xa4.* At 14 days after inoculation (DAI), the improved parent lines and F1 hybrid introgressed with *Xa4+Xa21* exhibited resistance to moderate resistance to all *Xoo* isolates with shorter lesion length (LL) comparable to IRBB52 that contained *Xa4+Xa21.* A significant difference was noted between the original and improved parent lines and hybrids in terms of disease reaction. The improved parent lines and hybrid have increased resistance to all the isolates tested, which was comparable to that of the resistant check containing *Xa4+Xa21.* This suggests that the introgression of the resistance gene *Xa21* was successful. The dissimilarity on morpho-agronomic traits between the original and improved

lines was detected, but could be minimized through further backcrossing. The improved parent lines could be used in hybrid line development while the hybrid PR40638H is ready to be deployed in BLB-affected areas.

Keywords: Hybrid, BLB, Xa gene, gene pyramiding, *Xanthomonas oryzae* pv. *oryzae (Xoo)*

INTRODUCTION

Heterosis or the increase effectiveness of certain trait on a hybrid is one major factor for a success in increasing crop yield. Hybrid rice shows effectiveness on yield production increasing it up to 15%. In addition to that, hybrid rice is more responsive to nitrogen fertilizers and exhibit vigorous growth. However, these attributes also makes it susceptible to various diseases [1].

Hybrid rice is more responsive to nitrogen fertilizer and exhibit vigorous growth. These attributes make most hybrids highly susceptible to the disease [2]. The practice of flag leaf clipping to facilitate out crossing in hybrid seed production further predisposes hybrid rice parent materials to infection [3].

Bacterial leaf blight (BLB) is one of the most devastating diseases of rice worldwide caused by the vascular pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). This bacterial pathogen can reduce grain yield of about 20-50% and up to 80% on severe cases [1, 4]. The threat of the susceptibility of the hybrid rice to this disease threatens the status of rice production in the near future. Deployments of rice cultivars with resistance genes have been proven as effective strategy to combat bacterial blight. However, early deployed rice cultivars are reported susceptible to bacterial blight. This could be caused by new strains of pathogen arising due to large scale and long-term cultivation. Furthermore, climate change and crop management process could trigger new strains that could overcome the resistance of rice cultivars [1].

Pyramiding of resistance genes intensifies the resistance of rice cultivars against bacterial blight. Pyramided genes offer broad-spectrum and higher resistance to the disease as compared to rice plant that contains only one resistance gene. Molecular markers allow easier selection of rice cultivars that contain the resistance genes, as compared to the conventional process of plant breeding where tedious pathotyping with different isolates is done, which is due the availability of resistance genes that are closely linked to known molecular markers. This method has become one of the effective ways in breeding of rice varieties with resistance [1, 5].Useful gene pyramiding has already been reported.

Using RFLP and PCR-based markers, Huang et al. [6] successfully pyramided four resistance genes *Xa4*, *xa5*, *xa13* and *Xa21*. In addition, three bacterial blight genes *xa5*, *xa13* and *Xa21* were successfully transferred into three promising lines as reported by Sanchez et al.[7]. Improved resistance with combined *xa5* and *Xa21* exhibited shorter lesions and showed better resistance as compared to those carrying single gene [8]. Similar results were obtained with higher levels of resistance in backcross lines pyramided with *Xa4* and *Xa21* genes from transgenic IR72 as reported by Khondker et al. [9] as cited by Tabanao et al. [1].

An elite hybrid rice, Mestiso 3 have been introgressed with the *Xa21* gene in addition to its originally contained *Xa4* gene, but was only tested to one isolate of *Xoo*, PXO79 representing race 3b [1]. Thus, this study evaluated the disease reaction of the original and improved parent lines and F1 hybrids against 14 isolates of *Xoo* and compared their important morpho-agronomic traits.

MATERIALS AND METHODS

Plant Materials

The following elite breeding lines and varieties were used in the study: improved lines (PR28A and PR39902-19R56) and F1 hybrid (PR40638H), along with original lines (IR68897A and IR60819-34-2R) and F1 hybrid NSIC Rc116H (Mestiso 3), differential IRBB lines: IRBB4, IRBB21 and IRBB52 (resistant checks) and IR24 (susceptible check). Seeds of test entries were obtained from the Plant Breeding and Biotechnology Division, Philippine Rice Research Institute (PhilRice), and International Rice Research Institute (IRRI). Each entry was pre-germinated for 48 hours at 28°C in water-soaked filter paper. Germinated seeds were planted individually in plastic seedling trays. Differential rice (IRBB) lines with a single gene (IRBB4 and IRBB21) and two genes (IRBB52) served as resistant checks and IR24 as susceptible check were used in this study.

Resistance Gene Confirmation by Molecular Markers

Genomic DNA was extracted by the CTAB (cetyl trimethyl ammonium bromide) method [10] from leaf tissue collected from single plants. Two gene-specific DNA markers, MP (F 5'-ATC GAT CGA TCT TCA CGA GG-3'; R 5'-TGC TAT AAA AGG CAT TCG GG-3') for Xa4 [1] and U1/I1 (F 5'-ATA GCA ACT GAT TGC TTG G-3'; R 5'-CGA TCG GTA TAA CAG CAA AAC-3') for *Xa21* (Tu et al. [11] as cited by Tabanao et al. [1]), tightly linked to the resistance genes *Xa4* and *Xa21*, respectively, were used to

confirm the presence of the R genes in each improved lines.

Polymerase chain reaction (PCR) was performed in atotal reaction volume of 8.0 µL, consisting of 0.8 µL 10x PCR buffer, 0.25 µL 50 mM MgCl2, 0.3 µL 5 mM dNTP, 0.5 µL 10 mM each of forward and reverse primers, 0.8 µL non-commercial *Taq* DNA polymerase, 3.55 µL sterile distilled water and 1.3 µL template DNA. The following temperature profile was used: initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 55 ^{o}C for 30 sec, and extension at 72 ^{o}C for 1 min, and a final extension at 72 ^{o}C for 8 min (Bio-Rad Laboratories Inc., T100^{TM} thermal cycler, USA).

Agarose gel (1.5%, 1.0×TBE, 150 V) and polyacrylamide gel (8% polyacrylamide, 1.0×TBE, 100 V) electrophoresis were used for the PCR products from U1/I1 primers and the PCR products from MP1 + MP2, respectively.The gels were stained and visualized with nucleic acid gel stain (GelRed ^{TM}, Biotium, Inc., USA), and documented using GelDoc^{TM} XR+ imaging system (Bio-Rad Laboratories, Inc., USA). Marker allele types of the genotypes were determined based on the unique band sizes as well as the banding patterns derived from PCR products (MP and U1/I1).

Evaluation of Bacterial Leaf Blight Resistance

The test materials were grown in the screen house of the Plant Breeding and Biotechnology Division at PhilRice Central Experiment Station. At maximum tillering stage, the plants were inoculated with the 14 isolates representing 10 Philippine races of *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* obtained from the IRRI using the leaf clipping method [12]. Inoculation was done in the late afternoon to reduce effect of other factors such as moisture and temperature on the aggressiveness of the pathogen. Proper irrigation was applied to avoid drought stress, which might interfere with bacterial blight symptoms. Agro-meteorological data showed that the conditions favored the pathogen requirements for disease development; the recorded relative humidity (RH) was above 70% and temperature ranging from 25ºC to 34ºC, and with zero total rainfall. Reactions of test entries to the disease wereevaluated 14 days after inoculation (DAI) by measuring lesion length (cm). The length of typically yellowish-grey lesions developed below the point of inoculation were measured in centimeter (cm) using a ruler. The average lesion length (LL) of all replicates per isolate per plant lines were considered to indicate the virulence level of a particular isolate on a

specific host genotype for that replicate. The reactions of resistance were expressed in lesion length (cm). Standard Evaluation System (SES) for rice [13] was used in scoring for disease reaction.

Characterization of Morpho-Agronomic Trait

Morpho-agronomic characters such as plant height (Ht), panicle number (PN), panicle length (PL), number of grains per panicle (NGP), spikeletfertility (SpFert), and 100-grain weight (100-GW) were gathered according to the SES for rice [13]. PH was measured from soil surface to tip of the tallest panicle (awns excluded). PL was measured as the average number in centimeters from the panicle neck to the panicle tip based on an evaluation of all the panicles from the plants. PN was the average number of panicles on the plants. NGP was calculated by counting the total number of filled and unfilled spikelets from the plants. SpFert was calculated as a percentage: the number of filled spikelets divided by the number of spikelets per panicle. 100-GW was measured in grams as the average weight of 100-filled grain from each plant.

Statistical Analysis

Data on lesion length were analyzed using analysis of variance (ANOVA), and comparison of means was done using Tukey's Honest Significant Difference (HSD) using SAS for Windows software, version 9.1 (SAS Institute Inc., Cary analyzed by analysis, NC, U.S.A. 2004). On the otherhand, T-test was used in mean comparison of morpho-agronomic data.

RESULTS AND DISCUSSION

Presence of Resistance Gene

Gene-specific PCR markers were used to confirm the presence of the resistance genes in each line. The expected PCR products from *Xa4* have a size of 160 bp and 1.4 Kb for the PCR products of *Xa21*. Figure 1 showed the DNA banding pattern and scoring based on the electrophoresis. Test entries with similar banding pattern with the resistant check were scored positive for the resistance gene (R), those with similar banding patters with the susceptible check were scored negative (S). The susceptible check, IR24 has no resistance genes and was expected to have less resistance. IRBB4 has*Xa4* resistance gene the same with the original Mestiso 3 hybrid and parent lines. IRBB21 contained *Xa21* resistance gene. The IRBB52 have two genes, *Xa4*+*Xa21*which is similar with the improved F1 hybrid PR40638H and parent lines.

Disease Reaction to *Xoo* Isolates

Listed in Table 1 is the lesion lengths obtained at 14 days after inoculation (DAI)

of 14 *Xoo* strains. Bacterial leaf blight was most severe in the susceptible check IR24, with lesion length ranging from 15.96 to 28.94 cm. However, the improved parent lines PR28A, PR39902-19R56, and F1 hybrid PR40638H, showed high levels of resistance from moderately resistant to resistant against 14 isolates. Lesion lengths in improved parent lines and F1 hybrid were consistently shorter, as compared to IRBB4 (with *Xa4* alone), and statistically comparable with IRBB21 (with *Xa21* alone) and IRBB52 (with *Xa4* + *Xa21*), when induce-inoculated with *Xoo* isolates. Figure 2 showed the lesions produced by selected *Xoo* isolates on test genotypes.

Multiple mean comparisons also detected no significant difference between the resistant check IRBB4, containing only *Xa4* resistance gene, and the original parent and hybrid lines of NSIC Rc116H, having similar resistance genes on most of the isolates inoculated in terms of disease reaction, except on isolates PXO99, PXO339, PXO349 and PXO363.

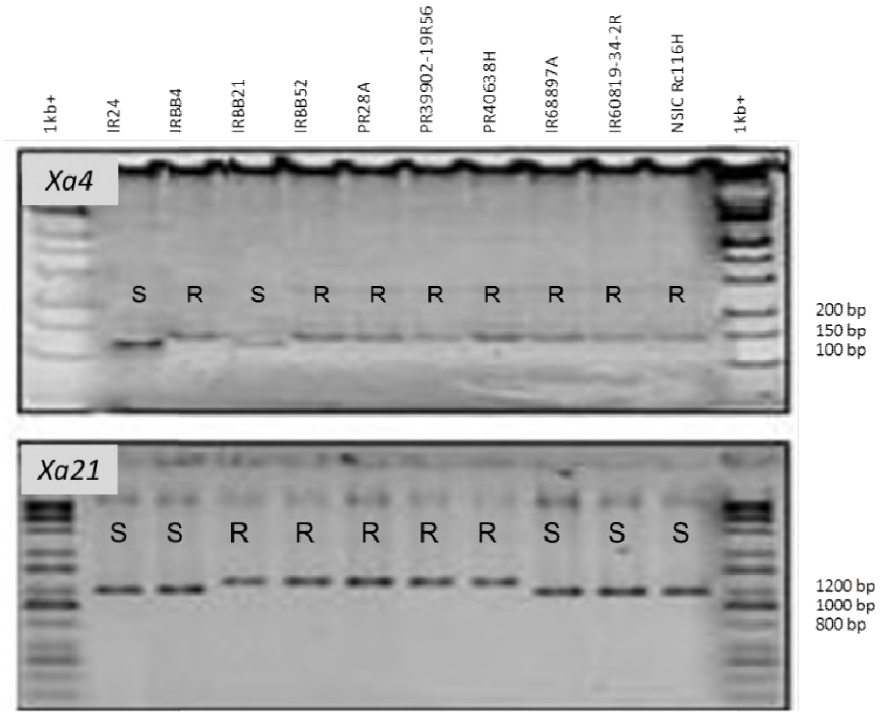

Figure 1: Resistance gene confirmation through PCR analysis. DNA amplified with primers MP1/MP2 linked with *Xa4*, and U1/I1 gene-based marker for *Xa21*. 1Kb DNA marker (Lanes 1 and 12),IR24 (Lane 2), IRBB4 (Lane 3), IRBB21 (Lane 4), IRBB52 (Lane 5), PR28A (Lane 6), PR39902-19R56 (Lane 7), PR40638H (Lane 8), IR68897A (Lane 9), IR60819R-34-2(Lane 10) and NSIC Rc116H (Lane 11). Legend: R – resistant & S – susceptible

Table 1: Relative levels of resistance reaction based onmean lesion lengths (cm) upon inoculation separately by 14 *Xanthomonas oryzae* pv. *oryzae* isolates under screen house conditions (February 2015).

Entries	Xa genes present	ISOLATES													
		PXO61	PXO86	PXO79	PXO340	PXO71	PXO112	PXO99	PXO145	PXO280	PXO339	PXO349	PXO347	PXO363	PXO341
IR24	None	24.06a± 1.5 (S)	15.42a± 8.0 (S)	17.86a± 3.1 (S)	21.41a± 2.2 (S)	21.07a± 3.5 (S)	15.96a± 2.5 (S)	23.93a± 0.7 (S)	21.60a± 1 (S)	24.31a± 2.7 (S)	18.60a± 4.6 (S)	22.71a± 1.3 (S)	21.39a± 4.6 (S)	24.96a± 3.2 (S)	28.94a± 1 (S)
IRBB4	*Xa4*	1.58b± 0.4 (R)	12.21ab± 2.2 (MS)	10.52b± 1.4 (MS)	16.89a± 1.7 (S)	5.35b± 0.4 (MR)	6.13b± 1.4 (MR)	13.79b± 1.7 (MS)	3.22b± 0.4 (R)	3.51b± 0.2 (R)	13.49ab± 2.2 (MS)	13.42b± 1.8 (MS)	17.42a± 0.6 (S)	10.72b± 2.1 (MS)	2.63c± 0.5 (R)
IRBB21	*Xa21*	1.90b± 0.3 (R)	2.16c± 0.4 (R)	2.09c± 0.4 (R)	2.61b± 0.8 (R)	2.39c± 0.5 (R)	3.66c± 1.2 (R)	3.73c± 2 (R)	3.09b± 0.1 (R)	3.91b± 1.5 (R)	4.56c± 3.1 (R)	3.20c± 0.5 (R)	2.59b± 0.4 (R)	3.18c± 0.7 (R)	15.86b± 0.6 (S)
IRBB52	*Xa4+Xa21*	1.54b± 1.1 (R)	6.31c± 6.6 (MR)	2.66c± 1.2 (R)	2.35b± 1 (R)	2.20c± 1.2 (R)	3.29c± 1.2 (R)	1.93c± 0.8 (R)	2.91b± 1.2 (R)	3.22b± 2 (R)	3.28c± 1.8 (R)	2.08c± 1.4 (R)	6.26b± 1 (MR)	3.33c± 2.8 (R)	2.07c± 0.4 (R)
IR68897A	*Xa4*	2.14b± 0.3 (R)	18.73a± 0.8 (S)	14.70ab± 3 (MS)	14.05a± 1.9 (MS)	8.07b± 0.5 (MR)	6.89b± 4.1 (MR)	19.00a± 2.5 (S)	6.31b± 3.1 (MR)	4.34b± 1.7 (R)	3.17c± 1.4 (R)	4.33c± 1.6 (R)	20.44a± 3.3 (S)	17.05a± 1.4 (S)	3.16c± 1 (R)
IR60819-43-2R	*Xa4*	4.61b± 3.7 (R)	14.18ab± 3.1 (MS)	10.06ab± 0.7 (MS)	12.18a± 2.6 (MS)	7.57b± 2.9 (MR)	7.80b± 1.4 (MR)	15.64b± 3.7 (S)	4.80b± 1.1 (R)	7.44b± 4.9 (MR)	4.73c± 0.6 (R)	3.64c± 0.2 (R)	18.40a± 4.4 (S)	18.07a± 5.8 (S)	3.82c± 1.5 (R)
NSIC Rc116H	*Xa4*	3.99b± 3.4 (R)	16.65a± 3.8 (S)	14.20ab± 4.4 (MS)	15.18a± 5.1 (S)	9.60b± 3.9 (MR)	9.63b± 2.3 (MR)	20.83a± 5.5 (S)	5.44b± 1.1 (MR)	6.79b± 3.2 (MR)	4.24c± 1 (R)	6.45c± 4 (MR)	16.88a± 6.3 (S)	20.77a± 6 (S)	6.15c± 2.7 (MR)
PR28A	*Xa4+Xa21*	0.69b± 0.1 (R)	5.37c± 2.6 (MR)	3.29c± 0.2 (R)	3.25b± 0.5 (R)	2.67c± 1.2 (R)	2.94c± 0.7 (R)	3.76c± 0.5 (R)	2.31b± 0.3 (R)	1.64b± 0.4 (R)	3.56c± 2.2 (R)	2.63c± 0.4 (R)	4.91b± 2.3 (R)	3.88c± 0.7 (R)	2.36c± 0.7 (R)
PR39902-19R56	*Xa4+Xa21*	2.05b± 1.2 (R)	1.90c± 0.0 (R)	5.34c± 0.3 (MR)	4.28b± 1.2 (R)	3.39c± 1 (R)	4.82c± 0.3 (R)	6.52c± 0.9 (MR)	4.42b± 1.1 (R)	4.92b± 2.6 (R)	3.69c± 1.6 (R)	4.13c± 1.7 (R)	9.51b± 0.8 (MR)	8.79c± 0.7 (MR)	2.33c± 1.6 (R)
PR0638H	*Xa4+Xa21*	0.98b± 0.1 (R)	4.79c± 0.30 (R)	2.23c± 0.6 (R)	3.51b± 0.6 (R)	1.68c± 0.3 (R)	2.94c± 0.5 (R)	2.59c± 0.3 (R)	2.54b± 0.4 (R)	1.89b± 0.4 (R)	2.63c± 0.5 (R)	2.43c± 0.7 (R)	5.21b± 3.2 (MR)	3.59c± 0.5 (R)	4.661c± 1.6 (R)

Lesion length(cm) measured 14 days after inoculation in maximum tillering stage

Data presented are the means of different treatments and replications with standard deviation. Means that do not share a letter (superscript) across different lines are significantly different at 5% level of significance.

LEGEND: R = Resistant (<5 cm), MR = Moderately Resistant (5.1 cm – 10 cm), MS = Moderately Susceptible (10.1 cm – 15 cm), S = Susceptible (>15.1 cm)

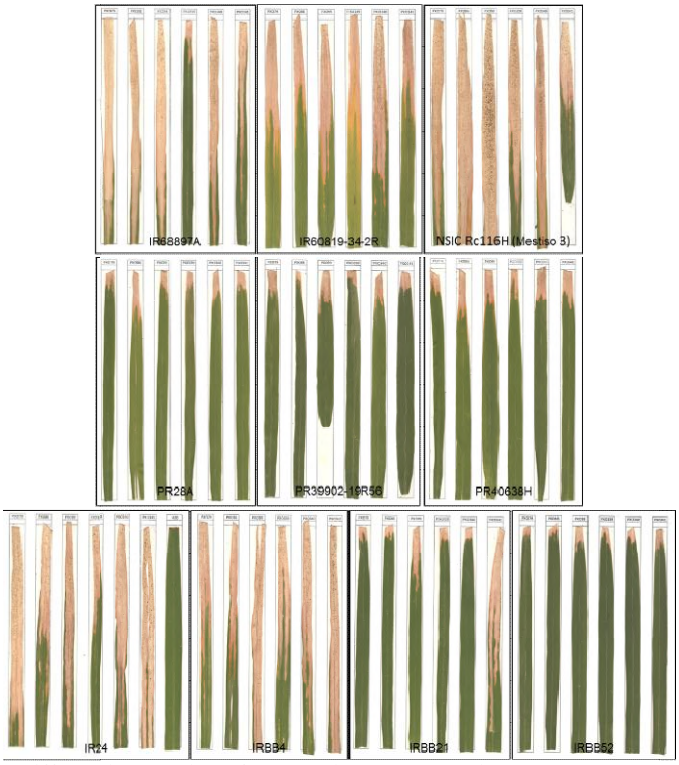

Figure 2: Differential reaction of test entries and IRBB lines to selected races of *Xoo*

The original CMS, restorer line and NSIC Rc116H have significant difference with their improved counterparts except on PXO145, PXO280, PXO339, PXO349 and PXO341 where the original parent lines and hybrid were scored resistant or moderately resistant to the isolates. Improved lines exhibited shorter lesion length that is comparable with the resistant check IRBB52 which means that the *Xa21* gene is successfully expressed.

Statistical analysis confirmed that the improved parent PR28A (improved CMS lines), PR39902-19R56 (improved restorer lines) and PR40638H (improved hybrid) have increased resistance to all the isolates tested which is comparable to that of the positive check IRBB52 (resistant check) containing the same resistance genes *Xa4+Xa21*, suggesting successful introgression of the resistance gene *Xa21*. Both PR28A and PR40638H exhibited short lesions when inoculated with most prevalent *Xoo* races in the Philippines. The presence of *Xa4+Xa21* genes for BLB tolerance was confirmed using molecular markers. This proved the successful introgression of BLB resistance genes on parent lines and F1 hybrid PR40638H. The combination of two or more *Xa* genes contributed to the increased level of resistance of the improved genotypes against bacterial blight [1]. Huang

et al. [6] reported similar results and claimed that this might be due to the interaction or complementation between the different resistance genes.

Morpho-Agronomic characteristics

Listed in Table 2 are the means for the morpho-agronomic traits of the original and improved CMS lines. The PR28A (improved line) are shorter, with 64.39 cm compared to 66.39 cm for IR68897A (original) in terms of height, while similar number of productive tillers were recorded for both lines. T-test revealed that there was no significant difference between the original and improved CMS lines in terms of their height and number of productive tillers.

Furthermore, means for the morpho-agronomic characteristics of the original and improved restorer lines such as, plant height, panicle number, panicle length, spikelet number per panicle, spikelet number per panicle and 100 grain weight are shown in Table 2. PR39902-19R56 (improved) showed inferior morpho-agronomic characteristics compared to the original IR60819-34-2R restorer line on all of the characteristics recorded. T-test suggests significant difference between most of the traits except on the number of grains per panicle. These differences between the original and improved lines could be due to incomplete backcrossing [1, 14]. Also linkage drag is a challenge during introgression where linked genes show reduction in fitness in a cultivar due to deleterious genes introduced along with the beneficial gene [15, 16]. Additional backcrossing could further recover the recurrent parent genome thereby minimizing the effect of linkage drag as suggested by Tabanao et al. [1].

PR40638H also showed inferior in terms of number of grains per panicle (NGP) compared to the original hybrid NSIC Rc116H. However, T-test showed that most traits between PR40638H and NSIC Rc116H are statistically similar.

Table 2: Morpho-agronomic performance of original and improved hybrids

Entry		Morpho-agronomic Characteristics					
		PH	PN	PL	NGP	SpFert	100-GW
CYTOPLASMIC MALE STERILE LINES	IR68897A (original)	66.39a	18a	-	-	-	-
	PR28A (improved)	64.39a	18a	-	-	-	-
RESTORER LINES	IR60819-34-2R (original)	86.72a	10a	25.05a	168.32a	84.67a	2.06a
	PR39902-19R56 (improved)	79.67b	8b	21.39b	159.22a	70.00b	1.99b
MESTISO 3 HYBRID	NSIC Rc116H (original)	104.00a	12a	24.70a	180.15a	71.67a	2.06a
	PR40638H (improved)	100.00a	12a	21.40a	159.22b	70.00a	2.02a

Significance at 5%. PH: Plant height, PN: Panicle number, PL: Panicle length, SpFert: Spikelet fertility, 100-GW: One hundred grain weight

CONCLUSION

Through artificial inoculation, this study confirmed that improved lines have broader resistance spectrum compared to the original hybrid lines. Thus, gene pyramiding increases bacterial leaf blight resistance. The improved parent lines could be used in hybrid line development while PR40638H can be deployed on bacterial leaf blight affected areas without the risk of losing yield. However, analysis of morpho-agronomic characteristics showed some dissimilarity but could be overcome by further backcrossing. Possibility of improving the resistance of hybrid parent lines by through MAS without risking the integrity of important morpho-agronomic characteristics was successfully demonstrated in this study. The improved parent lines could be used in hybrid line development and candidate for PVP licensing. The commercial release of this disease resistant hybrid PR40638H would greatly contribute to more stable yields in lowland rice areas prone to bacterial light in the Philippines.

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