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Understanding the Bacterial Blight Pathogen-Combining Pathotyping and Molecular Marker Studies

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Abstract: Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* has been and is one of the major constraints for rice production by virtue of its greater adaptation and pathotype variations since, its identification in the early seventies. The pathogen population being very dynamic is changing at an accelerated pace and posing a serious challenge to the agricultural sector. The present study was undertaken to understand and gain an insight on the prevalent pathogen population which is a prerequisite for deployment of the right combination of resistant genes to combat the pathogen population. Infected leaf samples were systematically collected from a hotspot (Maruteru) of the disease and were characterized by following both field inoculation pathotyping and at molecular level by DNA fingerprinting. Greater variation was observed in the molecular phenotypes than in virulence patterns. Among genes and their combinations studied, the four gene combination (*Xa4*+*xa5*+*xa13*+*Xa21*) was found more resistant against the isolates. Such observations open up new strategies and paradigms for aiming at focused breeding programs for more stable and durable resistant cultivars by pyramiding favorable genes together.

Key words: *Xanthomonas oryzae* pv. *oryzae*, bacterial blight, gene- combinations, phenotypes, pathotype, virulence

INTRODUCTION

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases prevalent in the major rice growing countries of Asia (Wang *et al*., 2006). In India, BB is a serious problem under irrigated and high fertilizer input conditions, which are conducive for disease development. Yield losses as high as upto to 80% have been reported under severe epidemics. Breeding for disease resistance is the most effective and economical method for control of BB that has a neutral impact on the environment. The BB is characterized by a high degree of race-cultivar specificity.

The pathogen is known to exhibit pathogenic variation, with diverse pathotypes or races in different rice growing areas. Many resistant genes have been identified and genetically defined in cultivars and germplasm. A clear understanding of the molecular mechanisms in host resistance to pathogens is a prerequisite for better design of control strategies for rice bacterial blight (Dai *et al*., 2007). There are over 30 reported races of isolates from several countries (Mew, 1987; Noda *et al*., 1996, 2001; Adhikari *et al*., 1999b; Shanti *et al*., 2001).

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Thirty two different BB resistant genes have been identified from wild and cultivated species of rice and designated in series from *Xa1* to *xa 32t* (Sun *et al*., 2003, 2004; Iyer and Couch, 2004; Gu *et al*., 2005; Chu *et al*., 2006; Jiang *et al*., 2006a, b; Xiang *et al*., 2006; Wang *et al*., 2006; Jin *et al*., 2007). Several donor genotypes carrying diverse genes for BB resistance have been used to develop BB resistant varieties (Khush *et al*., 1989). Pyramid lines have displayed higher levels and/or wider spectra of resistance to BB than the parents or NILs with single resistance genes, suggesting synergism and complementary gene action among resistance genes (Adhikari *et al*., 1999a; Huang *et al*., 1997; Narayanan *et al*., 2002; Shanti and Shenoy, 2005; Sundaram *et al*., 2008). However, the manifestations of resistance genes vary across locations due to geographic adaptations and structuring of the pathogen.

Various molecular tools like Restriction Length Fragment Polymorphism (RFLP) have been developed to determine the pathogen population structure of *Xanthomonas oryzae* pv. *oryzae* (Leach *et al*., 1992; Nelson *et al*., 1994; Ardales *et al*., 1996). To expidate the analysis of a large number of isolates a simple and efficient method of DNA fingerprinting based on PCR using primers corresponding to the repetitive element IS1112 have been developed (George *et al*., 1995). Outwardly directed oligonucleotides complementary to each end of the IS 1112 element were used to fingerprint pathogen strains, resulting in discrimination of different strains both in the Phillipines and India (George *et al*., 1995, 1997; Shanti *et al*., 2001).

Though a lot of research has been done on the R genes and pyramiding into different cultivars of interest, there is not much study on the pathogen population especially in India, where there is a diverse geographical variation within the country. Knowledge of the pathogen structure and changes in the races of the contemporary pathogen are very vital areas of research. The present study is part of a regional effort to apply knowledge of pathogen population structure to the deployment of resistance genes. It has been undertaken to identify genes and gene combinations conferring resistance to the contemporary BB pathogen of Maruteru (hotspot) and for deploying of such genes in future breeding programs so as to incorporate durable levels of resistance in promising high yielding varieties.

MATERIALS AND METHODS

Materials Used

All the genotypes used in this study were obtained from Central Rice Research Institute, Cuttack under the Asian Rice Biotechnology Network (ARBN) (Table 1). The experiments were initiated in kharif 2007 and repeated for three consecutive kharif seasons. The seeds were sown in nursery beds during first fortnight of June at Barwale Foundation research farm, Hyderabad, India. Twenty one day old seedlings were transplanted in the fields with a spacing of 20×20 cm. Standard package of practices were followed for raising the crop.

Genotypes used in the study were the near Isogenic Lines (NILs) for BB resistance genes in IR 24 genetic background viz., ARBN 120 (*Xa4*), ARBN 121 (*xa5*), ARBN 126 (*xa13*), ARBN 128 (*Xa21*) and checks including susceptible TN1, Karuna and resistant Malagkit Sung Song. Each NIL carried a defined resistance gene backcrossed into susceptible cultivar IR 24 (Ogawa *et al*., 1991). In addition, the response of a set of two, three and four gene pyramids ARBN 129 (*Xa4*+*xa5*), ARBN 130 (*Xa4*+*xa13*), ARBN 131 (*Xa4*+ *Xa 21*) ARBN 132 (*xa5*+*xa13*), ARBN 133 (*xa5*+ *Xa 21*), ARBN 134 (*xa13*+*Xa21*), ARBN 135 (*xa5*+ *xa13*+ *Xa21*) and ARBN 137 (*Xa4*+*xa5*+*xa13*+*Xa21*) in IR 24 background were also evaluated.

Collection of Infected Leaf Samples

Infected leaf samples were collected from farmers' fields adjoining Agricultural Research Station (ANGRAU), Maruteru which is a hotspot for bacterial blight disease in coastal Andhra Pradesh. For this purpose, the infected field was divided into five hills in the form of a W. Two infected leaf samples were collected from each hill. This was done in order to cover the entire infected field. A total of twenty five infected leaf samples were collected from infected fields.

Isolation and Maintenance of the Pathogen

All strains of *Xoo* were isolated in Barwale Foundation's molecular biology laboratory and maintained on modified Waikimoto's medium (Karaganilla *et al*., 1973) containing 20 g sucrose, 5 g peptone, 0.5 g calcium nitrate, 1.82 g disodium hydrogen phosphate, 0.05 g ferrous sulphate, 18 g agar-agar per liter with a pH 6.8 to 7.0. One colony was picked up per leaf sample and streaked onto a petri plate. For long term storage cultures were maintained in sterile distilled water at 4°C. When necessary these stored cultures were revived on modified Waikimoto's medium.

Evaluation of Resistance

Top five or six leaves of plants at maximum tillering stage were clip-inoculated with a cell suspension of 10^8 colony forming units mL^{-1} prepared from 48 h old cultures (Kauffmann *et al*., 1973). For each culture-strain combination, five leaves of a plant were inoculated per replication. A total of three replications were undertaken. Observations were recorded 15 days after inoculation and lesion lengths were measured to the nearest centimeter for classifying the disease response. Individual plants were classified as resistant (0-4 cm) and susceptible (>4 cm).

Genomic DNA Isolation

For DNA extraction, cultures were grown in 15 mL nutrient broth (per liter 10 g peptone and 3 g beef extract, pH 6.5) at 28°C on a rotary shaker (200 rpm). Genomic DNA of *Xoo* was extracted from 5 mL nutrient broth cultures grown overnight. The bacterial cells were pelleted and lysed in 650 μL extraction buffer [100 mM Tris pH 8, 100 mM EDTA, 250 mM NaCl, 15% SDS (w/v), 1% PVP-40 (w/v)] at 65°C for 30 min. DNA was isolated using a modified method with 100 μL of potassium acetate (3 M potassium and 5 M acetate) and precipitated with isoprgopanol (George *et al*., 1997).

PCR Profiling

Amplification was performed in a 25 μL volume containing 50 pM of the two *IS1112* based primers JEL1 and JEL 2, 20 ng of template DNA, 185 μM of dNTPs 2 units of Taq polymerase in a standard incubation buffer supplemented with 10% DMSO (v/v) and 0.75 μL of 1 M Tris HCl (pH 9.5) (George *et al*., 1995). The thermo profile used includes 1 min denaturation at 94°C followed by 30 cycles of denaturation at 94°C/10 sec, annealing for 1 min at 62°C followed by extension at 65°C for 8 min and a final extension for 8 min at 65°C using a thermo cycler (MJ Research Inc., USA). The amplified products were run on 6% polyacrylamide gel, visualized by silver staining procedure and documented. Banding pattern of each isolate was recorded in binary form and a dendrogram was constructed employing sequential, agglomerative, hierarchical and nested unweighted pair group method using arithmetic averages (SAHN/UPGMA) clustering routine of NTSYS-pc using the unweighted pair-group method using arithmetic averages.

RESULTS

The single genes and their gene combinations were chosen for this study based on our earlier studies as well as by studies conducted at All India Coordinated Rice Improvement Program (Shanti *et al*., 2001; Shanti and Shenoy, 2005). The results based on the mean disease reaction observed among the 16 genotypes to isolates exemplified that no single gene confers complete resistance to all the isolates (Table 1). Incidentally, individual genes showed susceptibility as compared to the gene combinations. Among the four single genes used in the study to evaluate their disease spectrum, the dominant gene *Xa21* showed a higher degree of resistance being resistant to ten isolates whereas *Xa4* showed the lowest degree of resistance being resistant to only five of the twenty five isolates. *xa5* and *xa13* were found moderately resistant with *xa5* being resistant to seven and *xa13* resistant to eight isolates. Figure 1 shows the varying resistance/susceptibility reactions of the 25 isolates to the 16 lines.

The two gene combinations performed better than single genes. Interestingly, *Xa4*+*Xa21* combination performed well (resistant to 20 isolates). This is in complementation with the earlier studies showing that *Xa4* though referred to as a defeated gene, it was found that this gene when in combination with *Xa21* showed higher level of resistance (Jeung *et al*., 2006). The next best combination was *xa5*+ *Xa21* and *xa5*+*xa13* showing resistance to eighteen of the twenty five isolates inoculated upon. The gene combination *Xa4*+*xa13* showed resistance to seventeen isolates. The gene combination having the least resistance was *xa13*+*Xa21* showing resistance to fifteen isolates only.

Table 1: Reaction of rice genotypes to isolates of *Xanthomonas oryzae* pv. *oryzae*

	Isolates																							
Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Malagkit Sung Song	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
IRBB 4	R	S	S	S	R	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
IRBB 5	R	S	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	
IRBB 13	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	
IRBB 21	R	R	S	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	
Xa4+xa5	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
Xa4+xa13	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	R	R	S	S	S	R
Xa 4+ Xa 21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	S	S
xa5+xa13	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
xa 5+ Xa 21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
xa13+Xa21	R	R	R	R	R	R	R	R	S	S	S	S	S	R	R	R	R	R	R	R	S	S	S	S	S
xa5+xa13+ Xa21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	S	S	R	R
Xa4+xa5 xa13+Xa21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Karuna	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
TN1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
IR 24	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

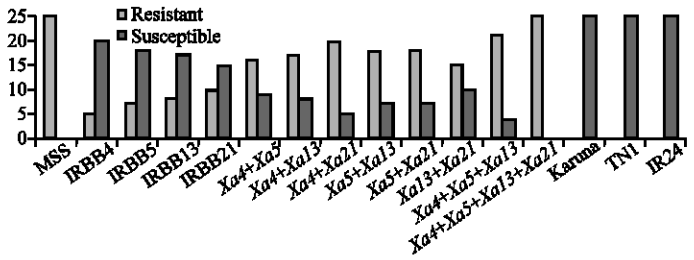

Fig. 1: Reaction of isolates against near isogenic lines and check cultivars

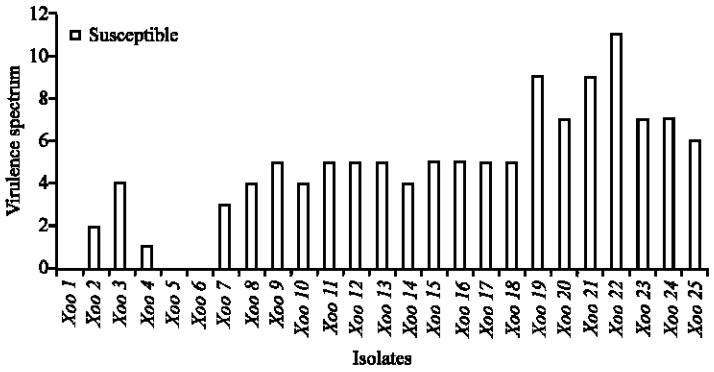

Fig. 2: Virulence spectrum of the 25 isolates

The three gene combinations showed some degree of susceptibility being susceptible to four isolates, but the four gene combination showed complete resistance to all 25 isolates (<1.0 cm lesion length) inoculated upon.

Figure 2 shows the virulence spectrum of the isolates used in the study. No isolate showed complete virulence. All the isolates showed varying degrees of resistance and susceptibility. The most virulent isolate was *Xoo 22* which was very virulent against twelve out of the thirteen different combinations (single, two, three and four gene). Only the four gene combination could not be knocked down. The next most virulent isolates were *Xoo 19* and *Xoo 21* showing virulence to nine lines. The least virulent isolates were *Xoo 1, Xoo 5* and *Xoo 6* which could cause disease only in the susceptible checks. They could not breakdown the resistance of even the single gene differentials.

Pathotyping Studies

Virulence analysis exhibited a high level of diversity among the different isolates. Using a differential set consisting of near isogenic lines and checks the twenty five strains were grouped into pathotypes. Field inoculations on the near isogenic lines and the check cultivars showed a total of seven pathotypes based on the resistant and susceptibility spectrum to each of the near isogenic line containing the individual genes (Table 2). Of these pathotypes XA 7 was the most prevalent with fourteen isolates falling into this category. This pathotype was the most virulent also by knocking down all the individual genes. The next most prevalent pathotype was XA 2 having four isolates in this class. The XA 1 had three isolates in its group and all the other pathotypes were represented by one isolate each. The isolates in XA 1 were the least virulent unable to knock down any of the single genes used in this study.

DNA Fingerprinting Studies

DNA fingerprinting of 25 isolates using the primer IS1112 showed distinct banding patterns among groups (Fig. 3). DNA profiles consisted of 40 scorable bands. Subsequent SAHN/UPGMA clustering of pathogen strains grouped them into 17 lineages at 95% level of similarity (Fig. 4). The maximum number of isolates in one lineage was four. At 82%, level of similarity there was no difference and all the isolates formed one group.

DNA fingerprinting showed 17 lineages and 18 haplotypes at 95% level of similarity. Field evaluation and virulence analysis detected seven pathotypes. Table 3 shows the comparison between molecular studies and field studies used in classifying the isolates. The

Table 2: Reaction of isolates to the host differentials

Pathotype	Isolates under each pathotype	*Xa4*	*Xa5*	*xa13*	*Xa21*
XA 1	*Xoo 1,5,6*	R	R	R	R
XA 2	*Xoo 4,23,24,25*	S	R	R	R
XA 3	*Xoo 2*	S	S	R	R
XA 4	*Xoo 8*	S	S	S	R
XA 5	*Xoo 10*	R	S	S	S
XA 6	*Xoo 7*	R	S	S	R
Xa 7	*Xoo 3, 9, 11, 12, 13, 14,15,16,17, 18,19,20,21,22*	S	S	S	S

Table 3: Comparison of lineages (DNA fingerprinting) versus pathotpyes (field studies)

Lineages	Isolates under each	Pathotype	Isolates under each pathotype
1	*Xoo 1*	XA 1	*Xoo 1,5,6*
2	*Xoo 4*	XA 2	*Xoo 4,23,24,25*
3	*Xoo 11*	XA 3	*Xoo 2*
4	*Xoo 20,21,22, 23, 24*	XA 4	*Xoo 8*
5	*Xoo 19, 25*	XA 5	*Xoo 10*
6	*Xoo 9*	XA 6	*Xoo 7*
7	*Xoo 15,17*	XA 7	*Xoo 3, 9,12,12,13,14,15, 16, 17, 18, 19, 20,21,22*
8	*Xoo 2*		
9	*Xoo 5,13,16*		
10	*Xoo 12*		
11	*Xoo 3*		
12	*Xoo 18*		
13	*Xoo 6*		
14	*Xoo 14*		
15	*Xoo 7*		
16	*Xoo 10*		
17	*Xoo 8*		

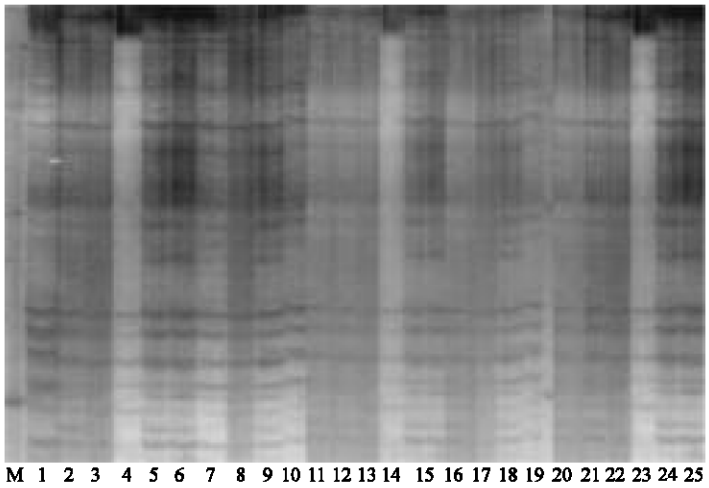

Fig. 3: DNA profile of *Xoo* isolates studied on PAGE using IS1112 primer

relationship between haplotypes, lineages and pathotypes appears to be a complex one, as observed in earlier studies (Shanti *et al*., 2001; Shanti and Shenoy, 2005; Nayak *et al*., 2008). Thus, although DNA fingerprinting is useful in revealing genetically related strains, there is no perfect prediction for virulence characterization. An example of this is *Xoo 5, 13* and *16* belong to one lineage at the molecular level, but at the field *Xoo 5* belonged to XA 4 and *Xoo 13* and *16* belonged to XA 8. Thus, one should judiciously integrate field studies and molecular studies and make the right choice of isolates to be used in breeding programs.

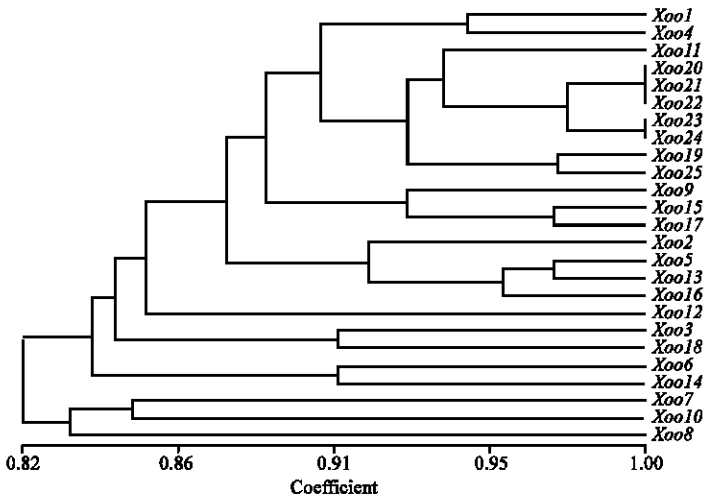

Fig. 4: SHAN/UPGMA Clustering of *Xoo* isolates based on DNA profiles using the primer IS1112

DISCUSSION

In the present study, the potential genes and their combinations conferring resistance to the contemporary BB pathogen have been identified. In addition the most prevalent and virulent pathogen population has been identified which in turn paves the way for the right choice of genes to be deployed.

Evaluation of single genes has enabled us to analyze the risk potential involved in deploying single genes for incorporation of resistance e.g., *Xa21*, had a broad spectrum of resistance (Wang *et al*., 1996) and presumably effective in many regions. Earlier reports also show that higher frequencies of virulent strains against the gene were noticed (Shanti *et al*., 2001). Such observations pave the path for selection of suitable gene combinations for pyramiding for longer lasting and durable resistance.

No single gene was able to provide complete resistance to all the isolates. This is in line with our earlier studies conducted to study the pathogen population in Eastern India (Shanti *et al*., 2001) and studies conducted elsewhere. The two gene combination and three gene combinations conferred a broader spectrum of resistance as compared to the single genes. Earlier studies conducted (Adhikari *et al*., 1999a; Shanti *et al*., 2001; Shanti and Shenoy, 2005; Joseph *et al*., 2004; Sundaram *et al*., 2008; Suh *et al*., 2009) have shown similar trends. It has been found that the presence of *Xa21* in combination with genes *Xa4, xa5* and *xa13* performed better than when treated alone. Though *Xa 4* individually did not perform well in complementation with *Xa21* it showed enhanced resistance. The three gene combination without *Xa4* showed susceptibility to four isolates whereas the four gene combination *(Xa4+xa5+xa13+Xa21)* has consistently shown positive results thereby proving that it is the right combination for gene pyramiding to combat the contemporary bacterial blight pathogen in South India.

DNA fingerprinting analysis has opened up new avenues for plant pathologists across the world since, this comprehensive analysis enables us to select representative pathogen strains to identify the appropriate resistance genes for use in the region. There is no need to inoculate thousands of isolates which is very tedious and cumbersome. Once a set of

representative isolates have been identified these can serve to inoculate the advanced breeding lines in any marker-assisted selection program. This saves on time, labor, money and seasons for forwarding of advanced material.

All the isolates have been collected from farmers' fields from one area only, but at the molecular as well as the field level they are showing a large amount of variability. This is clearly indicative of the fact that many races or strains or pathotypes of *Xoo* can occur in a given location, of which a particular strain may be more virulent. All the isolates which are weakly susceptible and unable to breakdown the resistance of the differentials can be avoided and only the very and moderately virulent ones can be incorporated in the breeding programs in order to get the best results.

Xoo 22 showed very high virulence by breaking down the three gene combination also. Two other isolates *Xoo 16* and *Xoo 21* though not as virulent as *Xoo 22* were also able to defeat the three gene combination. At the molecular level *Xoo 21* and *Xoo 22* belong to the same lineage. At the field level all three belong to the same pathotype. In an asexually propagated pathogen, like bacteria in absence of recombination it acquires favorable mutations one at a time. By accumulating favorable mutations in series, they tend to develop co-adaptive gene complexes. Once a favorable combination of genes has evolved, asexual reproduction and selection ensures the maintenance of the rapid dissemination of that particular combination. There is every possibility that all the isolates belonging to XA 7 will very soon evolve and breakdown the three gene combination also. Hence, the four gene combination is more apt for providing durable and longer lasting resistance.

The information gained in this study has significant implications for regional gene deployment. Though, lot of research has been conducted on pyramiding there is not much information available on the pathogen spectrum. The pathogen's ability to rapidly overcome major genes (Mew, 1987; Mew *et al*., 1992) is one of the major challenging areas of research for deployment of R gene containing lines in order to maximize the durability spectrum in the target location (Adhikari *et al*., 1999a). The recent advances in cloning and characterization of major genes (Dai *et al*., 2007) have enabled incorporation of major genes for resistance to specific races of the pathogen.

In this study, a set of NILs having single, two, three and four gene combinations have been evaluated. Field inoculated data indicated the effectiveness of some of the individual genes with broad spectrum of resistance shown by *Xa21* followed by *xa13*. Among the two gene combinations the broadest spectrum of resistance was shown by *Xa4+ Xa21* followed by the combinations *xa5+xa13* and *xa5+ Xa 21*. The three gene combination showed a broad spectrum of resistance being susceptible only to four isolates. The resistance spectrum of the two, three and four gene combinations may presumably be due to the complementary action of the resistance genes (Yoshimura *et al*., 1995). Similar observations were made in studies conducted in China and Nepal (Zheng *et al*., 1998; Adhikari *et al*., 1999a; Ming *et al*., 2006; Sundaram *et al*., 2008).

In conclusion, the present study indicates that the effectiveness of genes vary in different regions. For longer lasting and durable resistance regional information of individual genes (Shanti and Shenoy, 2005; Nayak *et al*., 2008; Suh *et al*., 2009) is a prerequisite which in turn can pave the way for deployment of the right gene combinations. In addition the intensified pathogen studies have shown the prevalence of one particular pathotype. Representative isolates from each group can be used to inoculate the advanced breeding lines and test their response to the disease. These results shed light on the right kind of gene combinations to be used in the target areas so as to insulate the losses caused due to BB and combat the pathogen population using broad spectrum and durable resistance.

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