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Pyramiding blast, bacterial blight and brown planthopper resistance genes in rice restorer lines

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Abstract

Rice blast, bacterial blight (BB) and brown planthopper (BPH) are the three main pests of rice. This study investigated pyramiding genes resistant to BB, blast and BPH to develop restorer lines. Ten new lines with BB, blast and/or BPH resistance genes were developed using MAS (marker-assisted selection) and ATS (agronomic trait selection) methods. Only HR13 with resistance genes to BB, blast and BPH was obtained. In addition to BB and blast resistance, four lines (HR39, HR41, HR42 and HR43) demonstrated moderate resistance to BPH, but MAS for BPH resistance genes was not conducted in developing these four lines. These data suggested that there were unknown elite BPH resistance genes in the Zhongzu 14 donor parent. A more effective defense was demonstrated in the lines with *Pi1* and *Pi2* genes although the weather in 2012 was favorable to disease incidence. Blast resistance of the lines with a single resistance gene, *Pita*, was easily influenced by the weather. Overall, the information obtained through pyramiding multiple resistance genes on developing the restorer lines is helpful for rice resistance breeding.

Key words: rice, blast, bacterial blight, brown planthopper, resistance, pyramid

1. Introduction

Rice (*Oryza sativa* L.) is a staple food crop in China that feeds more than 60% of the population, and it contributes nearly 40% of the total calorie intake (Cheng *et al.* 2007). Compared with conventional varieties, hybrid rice can significantly increase rice yields and has made a large contribution to the self-sufficiency of the food supply in China. However, most of the hybrids released do not have resistance to specific biotic stresses (Khush and Jena 2009).

Rice blast, bacterial blight (BB) and brown planthopper (BPH) caused by *Magnaporthe grisea*, *Xanthomonas oryzae pv. oryzae* (Xoo) and *Nilaparvata lugens* Stål, respectively, are the most destructive diseases and insects causing significant reduction in rice production throughout China and in other Asian rice-growing countries. Rice blast alone can cause annual yield losses of between 10 and 30% of the total harvest, and its occurrence was reported by the Ministry of Agriculture of China to be as high as 20% of the hybrid rice fields cultivated in 2006 (Jiang *et al.* 2012). BB disease, in its severe form, is known to cause yield losses ranging from 74 to 81% (Srinivasan and Gnanamanickam 2005). The damage caused by BPH feeding has the greatest effect on the growth and crop yield of the susceptible rice plant through the removal of assimilates

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and the reduction in photosynthetic rate of leaves, ultimately causing plant death in its severe form (Jirapong *et al.* 2007). Deployment of host plant resistance is considered to be the best option for managing the diseases and insects. Breeding rice varieties with multiple disease and insect resistance genes will broaden the resistance spectrum and increase the resistance durability for the varieties.

With the development of gene identification technologies, the marker-assisted selection (MAS) technique is typically used to improve disease and insect resistance. The scope of MAS breeding for targeted introgression of BB resistance genes (Huang *et al.* 1997; Chen *et al.* 2000; Chen *et al.* 2001; Sundaram *et al.* 2008, 2009), blast resistance genes (Amante-bordeos *et al.* 1992; Hittalmani *et al.* 2000) and BPH resistance genes (Sharma *et al.* 2004; Jena *et al.* 2006) has been successfully demonstrated. In addition, the introgression of two different disease or insect resistances has been conducted (Jiang *et al.* 2004). However, to the best of our knowledge, there is no report on the simultaneous introgression of BB, blast and BPH resistance into the lines of hybrid rice.

Currently, the production of hybrid rice is primarily based on the three-line hybrid system, which involves a cytoplasm male sterile (CMS) line, a corresponding isonuclear maintainer line, and a genetically diverse restorer line. In addition, the sterile line is maintained by being crossed with its maintainer line, and hybrid seed is produced by crossing the sterile line with the restorer line (Cheng *et al.* 2012). Generally, restorer lines are much easier to be improved through breeding techniques than sterile lines because no sterility is considered. Shuhui 162 and Zhongzu 14 are two restorer lines in hybrid rice. Shuhui 162 is resistant to only blast. Zhongzu 14 is resistant to BB, blast and BPH, but its resistance gene to BB is recessive, which cannot demonstrate its resistance in heterozygous-genotype hybrid rice. Hence, in this study, new restorers with multiple resistances to diseases and BPH were developed using the MAS technique and further evaluated by artificial inoculation in two years. These results impart valuable information for breeding resistance in rice.

2. Results

2.1 Pyramiding of different resistance genes into new lines

The two crosses were conducted with the MAS technique and the ATS method. The status of these plants carrying heterozygous or homozygous resistance genes is shown in Figs. 1 and 2. After obtaining plants with all homozygous resistance genes, pedigree selection was used to breed elite lines. In cross 1, the MAS technique was used to identify 8 compound F_1-1 plants, 10 compound F_1-2 plants, 7 F_2 plants and 102 F_3 plants from compound F_1-1, compound F_1-2, F_2 and F_3 generations, respectively. The ATS method was further used to select 66, 50 and 35 lines to generate next generations from F_4, F_5 and F_6 populations, respectively (Fig. 1). One line from 15 F_7 lines (*PitaPitaXa23Xa23Bph3Bph3*) and three lines from 20 F_7 lines (*PitaPitaXa23Xa23bph3bph3*) were named HR13 and HR15, HR22, and HR34, respectively. In cross 2, the MAS technique was used to identify 5 F_2 and 24 F_3 plants from F_2 and F_3 generations. The ATS method was further used to select 20, 18, 15 lines to generate next generations from F_4, F_5 and F_6 populations, respectively (Fig. 2). Six lines from 15 F_7 lines were designated as HR39, HR41, HR42, HR43, HR45 and HR47.

Ten new lines containing BB, blast and/or BPH resistance genes were obtained (Table 3). The aim of cross 1 was to pyramid *Pita*, *Xa23* and *Bph3* genes together with the multiple crosses and MAS techniques. However, only one line, HR13, containing the three resistance genes was obtained. Another three lines were pyramided with BB and blast resistance genes. The aim of cross 2 was to introgress the *Xa23* gene into Zhongzu 14. Six lines pyramiding the *Xa23* gene

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with *xa5*, *Pi1* and *Pi2* genes were achieved. The ten newly obtained lines further restored the fertility of Xieqingzao A to a normal level in the F_1 generation.

2.2 Diseases and BPH resistances of the new lines obtained in two years

After the artificial inoculation of blast, BB and BPH in 2012 and 2013, the diseases and BPH resistance levels of the new lines were evaluated (Table 3). All of the lines showed high resistance to blast and BB. There was a small change in blast resistance between the two years as the blast resistance in 2012 was lower than that in 2013. The lines obtained from cross 2 showed a higher resistance to blast than the lines from cross 1 in 2012 because most of the lines with the *Pita* gene (from cross 1) had only moderate resistance (3-level) to blast and the lines pyramiding *Pi1* and *Pi2* (from cross 2) maintained high resistance (0- to 1-level). A more effective defense against blast was demonstrated in the new lines containing *Pi1* and *Pi2* genes.

Only four lines showed BPH resistance. HR13, the line containing the *Bph3* gene, showed moderate resistance (3- to 5- level in both years) to BPH. The four lines obtained from cross 2 demonstrated moderate resistance (3- level in both years) to BPH although they had no *Bph3* gene. These results suggested that Zhongzu14 might be the donor of BPH resistance demonstrated by the four lines.

2.3 The influence of weather on diseases

The meteorological data collected in 2012 and 2013 were compared (Table 4). The results showed that the humidity and rainfall were different between the two years. In 2012, the total rainfall from June to September was 184.00 mm, and the total rainfall in 2013 was only 88.10 mm. Therefore, the rainfall for this period in 2012 was 2-fold more than the rainfall in 2013. Furthermore, during the week following August 5th (Fig. 3), which was the day of the artificial inoculation of blast and BB, the humidity in 2012 was almost 2-fold more than that in 2013, and the rainfall was almost zero for the same week in 2013. Thus, the weather the week after inoculation in 2012 was favorable to disease incidence.

By combining the disease resistance with the weather difference in the two years (Tables 3 and 4 and Fig. 3), we showed that weather did not influence BB resistance (near 0level in both years). However, blast resistance differences (varying from 0 to 1 level or 0- to 3- level) in the two years was observed, which suggested that the weather might have some influence on the blast resistance of the lines. The influence of weather on BPH resistance was not analyzed because the BPH resistance evaluation was conducted in a greenhouse under controlled conditions.

3. Discussion

Diseases and insects are major biotic stresses that cause significant yield losses globally. With the development of a comprehensive molecular genetic map of rice, at least 83 major resistance genes for blast, 38 resistance genes for BB, and 27 resistance genes for BPH have been identified (China National Rice Data Center, http://www.ricedata.cn/gene). Gene pyramiding using molecular techniques for conventional breeding is now a common technology, especially in rice breeding for disease and insect resistance. Pyramiding of multiple resistance genes into a single genetic background leading to the simultaneous expression of more than one gene in a variety is a strategy to prevent or delay the breakdown of resistance as the probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is much lower than for a single gene (Mundt 1990). In our study, pyramiding genes for resistance to different diseases and BPH as well as pyramiding different genes resistant to one disease were performed.

Because resistance genes from restorer lines in a three-line hybrid rice display heterozygous genotypes, a completely dominant resistance gene with a broad resistance spectrum is needed (Ji *et*

al. 2014). The *xa5* gene, which is naturally found only within the *Aus* subpopulation of rice (Garris *et al.* 2003), provides recessive resistance to several *Xoo* races from the Philippines. Conversely, the *Xa23* gene has a broader resistance spectrum to different BB races, displays a high resistance level during all growth stages and is highly heritable (Zhang *et al.* 1998; Zhang *et al.* 2001). Zhou *et al.* (2011) determined that there is no genetic background effect on the expression of the *Xa23* gene, suggesting that *Xa23* is of great value in a hybrid rice breeding program with BB resistance. Hence, the ten lines in this study were introgressed with the *Xa23* gene donated by CBB23, and high BB resistance was demonstrated in the lines (Table 3). The ability of the new lines to restore fertility in CMS lines was further confirmed.

The blast resistance gene, *Pi1*, was originally identified in the cultivar LAC23 (Mackill and Bonman 1992), an upland cultivar from Liberia, and it has a broad resistance spectrum. Only 10.35% of strains of the 792 Chinese isolates collected in central and southern China could infect the near-isogenic line (NIL) C101 LAC, which contains the *Pi1* gene and the susceptible cultivar CO39 background (Chen *et al.* 2001). The *Pi2* gene was first introgressed from a highly resistant *indica* cultivar, 5173, into the susceptible cultivar, CO39 (Mackill and Bonman 1992). Extensive field tests in several countries have indicated that *Pi2* is one of the rice blast resistance genes with a broad resistance spectrum (Chen *et al.* 1996). *Pita* is a single copy resistance gene in which the resistance specificity is determined by a single amino acid (Wang *et al.* 2010). The *Pi-ta* resistance allele was introduced from the Asian "Tetep" landrace variety, which is resistant to all common races of the blast fungus (Jia *et al.* 2004). With the introgression of the genes, the blast resistance of the new lines was demonstrated in our study. The effect of pyramiding *Pi1* and *Pi2* was similar to that of the *Pita* gene in 2013. However, in 2012, the lines with *Pi1* and *Pi2* showed higher blast resistance than the lines with the *Pita* gene. The weather of the first week after inoculation in 2012 had higher humidity and rainfall (Fig. 3), which was favorable to disease incidence. Certain cultivars show durable resistance because they "… remain resistant … even though they are extensively cultivated in environments favorable to disease" (Johnson 1981). Hence, a more effective defense was demonstrated in the lines with *Pi1* and *Pi2* genes.

Deployment of resistant varieties carrying various resistance genes has been successful for BPH control. *Bph1, bph2, Bph3* and *bph4* (Sharma *et al.* 2004; Sun *et al.* 2006; Jirapong *et al.* 2010; Peñalver Cruz *et al.* 2011) have been used extensively. Rice cultivars carrying *Bph3* have shown a higher degree and a broader spectrum of resistance against BPH (Jirapong *et al.* 2007). Nevertheless, the new line, HR13, containing the *Bph3* gene introgressed from Rathu Heenathi (RH), showed a moderate resistance to BPH. There might have been a certain genetic background effect on the *Bph3* gene because only moderate resistance to BPH was demonstrated compared to that of the donor RH. In contrast, the four lines (HR39, HR41, HR42 and HR43) with BPH resistance from Zhongzu 14 maintained a resistance level of 3 in both years. It has long been proposed that moderate and/or polygenic resistance to insect pests, including BPH, should provide more durable resistance than single major genes (Heinrichs 1986; Bosque-Perez and Buddenhagen 1992). Though it is not clear about the BPH resistance genes of Zhongzu 14, a more effective defense against BPH was demonstrated in the new lines originating from Zhongzu 14. Further evaluation and gene mapping of the BPH resistance for Zhongzu14 is required to explore its resistance to BPH.

4. Conclusion

Overall, pyramiding the three different disease and BPH resistances into the rice restorer lines was successful. The lines introgressed with multiple resistance genes will prolong the planting

years of the new lines. The influence of weather on blast resistance should be considered for the stability of rice yields. Further evaluation of the resistance levels of hybrid rice using the lines is needed in future studies. The BPH resistance genes of Zhongzu14 will also be mapped in future studies. This study will help accelerate the application of MAS breeding in rice improvement.

5. Materials and methods

5.1 Plant materials and breeding strategy

Five parents were used to pyramid disease and BPH resistance into the new lines (Table 1). The Shuhui162 restorer line contains the *Pita* gene. The Zhongzu14 restorer line contains *Pi1*, *Pi2* and *xa5* genes, and it is resistant to BB, blast and BPH. The BPH-resistance gene donor RH contains the *Bph3* gene. CBB23 and HN88 contain the *Xa23* gene. HN88 originated from CBB23 and is a new restorer line with high productive-tiller-rate and thousand-grain-weight.

Two crosses, namely Shuhui162/CBB23//HN88///RH (cross 1) and Zhongzu14/CBB23 (cross 2), were conducted. After obtaining compound F_1 or F_1, self-pollination was continuously performed for several generations to make the resistance genes homozygous using the MAS technique and to stop other agronomic trait segregation through the ATS method and pedigree selection. Herein, the ATS method involves selecting agronomic traits of the progenies similar to the restorer parents by artificially judging for the background selection. Crosses between Xieqingzao A and the new lines were further conducted to evaluate their restoring fertility for CMS lines.

5.2 MAS technique

Six markers were used to select corresponding genes in the breeding of each generation (Table 2). DNA samples were extracted from fresh leaves using a simple one-step method (Ji *et al.* 2014). Leaves with a length of approximately 3 mm were immersed in Buffer A containing 100 mM Tris-HCl (pH 9.5), 1 mol L^{-1} KCl, and 10 mmol L^{-1} EDTA. The samples were crushed using a multi-sample tissue lyser (Jingxin Technology Co. Ltd., Shanghai, China), and the supernatants were collected by centrifugation at 4000 r min for 5 min for DNA amplification.

Polymerase chain reaction (PCR) was performed in a 15 μL reaction volume containing 0.8 μL of supernatant, 2× PCR Buffer (including Tris-HCl, KCl, and $MgCl_2$), 2 mmol L^{-1} dNTPs, 0.9 μmol L^{-1} primer pairs, and 0.3 U KOD FX polymerase (Toyobo Co. Ltd., Shanghai, China). The reaction mixture was initially denatured at 94℃ for 2 min followed by 30 cycles of PCR amplification with the following parameters: 10 s of denaturation at 98℃, 30 s of primer annealing at 50℃ (53℃ for marker C189), and 1 min of primer extension at 68℃. Finally, the reaction mixture was maintained at 68℃ for 7 min before completion. The amplified product was electrophoretically resolved on a 2% agarose gel using Gelrad staining for C189 and YL155/YL187, and it was also resolved on an 8% denaturing polyacrylamide gel using silver staining for RM122, RM224, (Indel) PI2-4 and RM589.

5.3 Disease and BPH resistance evaluation

After several successive segregating generations, new lines pyramiding multiple resistance genes were sown on June 5th and transplanted on June 26th in the field at the China National Rice Research Institute, Fuyang, China. Resistance to BB and leaf blast was evaluated by artificial inoculation on August 5th in the field. Isolates of the two diseases prevalent at the area were provided by Mr. Tao Rongxiang of Zhejiang Academy of Agricultural Sciences. The lines planted in the field were inoculated with BB disease isolates using the leaf-clipping method. Nine leaves of three plants were inoculated with BB pathogens, and lesion length (LL) was recorded for each leaf 25 days after inoculation. The heartleaf-injecting method was used during the middle of the

tillering stage to evaluate the level of blast resistance. Five heartleaves for each line were inoculated with blast pathogens, and the LL of blast infection was recorded two weeks after inoculation. The susceptible controls to blast and BB were Zhongzheyou 1 and Jingang 30, respectively.

A modified seedbox screening technique (MSST) was used to evaluate the BPH resistance. Seedlings of the lines at the same growth stage were planted for BPH infestation in a greenhouse. At the second-leaf stage, the seedlings were infested with 2nd to 3rd instar BPH nymphs at a density of ten insects per seedling. When 70% of the seedlings of the TN1-susceptible control were dead, the percent mortality of the lines was determined. The BPH resistance of the lines was evaluated with scores of 0, 1, 3, 5, 7 or 9 according to the criteria adapted from the International Rice Research Institute (IRRI 1988).

The BB, blast and BPH resistance evaluations were replicated with three plots.

5.4 Weather data collection

The rice lines grew to the heading stage in mid-August and matured in late September on the same farm for both years. A small weather station (Watchdog 2475, SPECTRUM Technologies, Inc.) was used to collect meteorological data, including temperature, rainfall and humidity, during the growth period from June 5th to September 30th in the two years. The data were collected by the station every half hour each day and were averaged for analysis.

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Table 1 Details of the five parents

Parents for crosses	Details
RH	Donor of *Bph3*
CBB23	Donor of *Xa23*
HN88	A restorer line containing *Xa23* gene
Shuhui162	A restorer line containing *Pita* gene
Zhongzu14	A restorer line containing *Pi1*, *Pi2* and *xa5* genes with multiple resistance to diseases and insects

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Table 2 Gene linked markers for disease and insect resistance genes

Gene	Donor	chromosome	Linked marker	Distance	Primer pair	Expected band sizes	Reference
Xa23	CBB23, HN188	11	C189	0.8cM	F:5’-TAAGTTCTACATCGACCCCA-3’ R:5’-CACATGAAGAGCTGGAAACG-3’	900bp	Wang *et al.*, 2005
xa5	Zhongzu14	5	RM122	0.4cM	F: 5’-GAGTCGATGTAATGTCATCAGTGC-3’ R: 5’-GAAGGAGGTATCGCTTTGTTGGAC-3’	227bp	Blair M W *et al.*, 2003
Pita	Shuhui162	12	YL155/YL87	0.0cM	F: 5’-AGCAGGTTATAAGCTAGGCC-3’ R: 5’-CTACCAACAAGTTCATCAAA-3’	1042bp	Wang Z *et al.*, 2007
Pi1	Zhongzu14	11	RM224	0.0cM	F: 5’-ATCGATCGATCTTCACGAGG-3’ R: 5’-TGCTATAAAAGGCATTCGGG-3’	157bp	Haichao Jiang *et al.*, 2012
Pi2	Zhongzu14	6	(Indel)PI2-4	0.0cM	F: 5’-CGGTAAGAGTAACACCAAGC-3’ R: 5’-GACGTGCGAGTTGTGACAGCT-3’	236bp	Haichao Jiang *et al.*, 2012
Bph3	RH	6	RM589	0.9cM	F: 5’-ATCATGGTCGGTGGCTTAAC-3’ R: 5’-CAGGTTCCAACCAGACACTG-3’	186bp	Jirapong *et al.*, 2007

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Table 3 Pyramiding disease and insect resistance genes and evaluation of resistance by artificial inoculation for the two years in the new lines

Description		Pyramiding resistance genes by MAS						Resistance results by artificial inoculation					
Line	Origin	Blast resistance gene			BB resistance gene		BPH resistance gene	Blast resistance level		BB resistance level		BPH resistance level	
		Pita	*Pi1*	*Pi2*	*Xa23*	*xa5*	*Bph3*	2013	2012	2013	2012	2013	2012
HR13	Shuhui162/CBB23//HN88///RH	+	-	-	+	-	+	0	1	0	1	3	5
HR15		+	-	-	+	-	-	1	3	0	0	9	9
HR22		+	-	-	+	-	-	0	3	0	0	9	9
HR34		+	-	-	+	-	-	0	3	0	0	9	9
HR39	Zhongzu 14/CBB23	-	+	+	+	+	-	0	0	0	0	3	3
HR41		-	+	+	+	+	-	0	1	0	0	3	3
HR42		-	+	+	+	+	-	0	1	0	0	3	3
HR43		-	+	+	+	+	-	0	1	0	0	3	3
HR45		-	+	+	+	+	-	0	1	0	0	5	5
HR47		-	+	+	+	+	-	0	1	0	0	9	9
RH		-	-	-	-	-	+	7	7	7	7	1	1
CBB23		-	-	-	+	-	-	7	7	0	0	9	9
Shuhui162		+	-	-	-	-	-	0	0	5	5	9	9
Zhongzu 14		-	+	+	-	+	-	0	0	0	0	3	3

"+" means that positive band is showed using the marker. "-"means negative band is showed using the marker. In BB resistance level column, LL < 1 cm means high resistant level (0 level) and 1.1 cm < LL < 3 cm means resistant level (1 level). For blast resistance level, 0 level (high resistant level) means no lesion was found; 1 level (resistant level) means that the size of the lesion was that of a needle head; 3 level (moderately resistant level) means that the lesion diameter was approximately 1-2 cm (Tao *et al.* 2006).

Table 4 Meteorological data from June to September (2012 and 2013)

	Humidity (%)	Temperature (℃)	Rainfall (mm)
Total in 2012	8948.18	3122.94	184.00
Total in 2013	7899.74	3265.06	88.10
Average in 2012	75.83	26.47	1.56
Average in 2013	66.95	27.67	0.75

 Doi: 10.1016/S2095-3119(15)61165-0

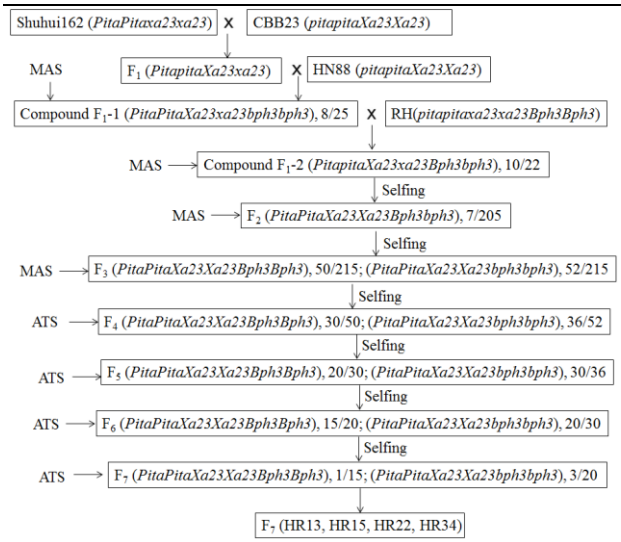

Fig. 1 Scheme of cross 1 showing the use of the MAS technique and ATS method to develop new restorer lines containing the *Pita*, *Xa23* and/or *Bph3* genes.

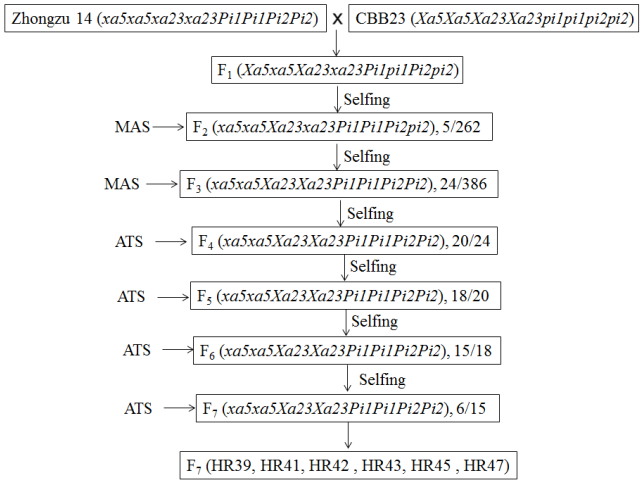

Fig. 2 Scheme of cross 2 showing the use of the MAS technique and ATS method to develop new restorer lines containing the *Pi1*, *Pi2*, *Xa23* and *xa5* genes.

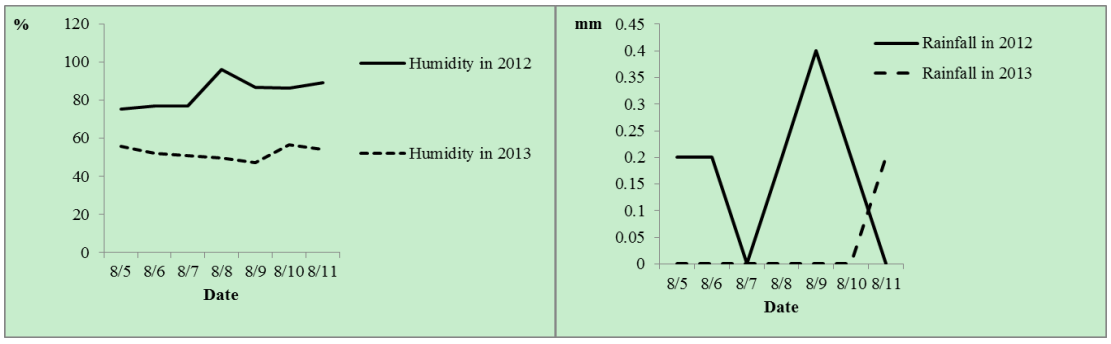

Fig. 3 Weather trends for one week after artificial inoculation of the two diseases.