



Plant biology and pathology/Biologie et pathologie végétales

Phenotypic screening and molecular analysis of blast resistance in fragrant rice for marker assisted selection

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ABSTRACT

Experiments were conducted to identify blast-resistant fragrant genotypes for the development of a durable blast-resistant rice variety during years 2012–2013. The results indicate that out of 140 test materials including 114 fragrant germplasms, 25 differential varieties (DVs) harbouring 23 blast-resistant genes, only 16 fragrant rice germplasms showed comparatively better performance against a virulent isolate of blast disease. The reaction pattern of single-spore isolate of *Magnaporthe oryzae* to differential varieties showed that *Pish*, *Pi9*, *Pita-2* and *Pita* are the effective blast-resistant genes against the tested blast isolates in Bangladesh. The DNA markers profiles of selected 16 rice germplasms indicated that genotype Chinigura contained *Pish*, *Pi9* and *Pita* genes; on the other hand, both BRRI dhan50 and Bawaibhog contained *Pish* and *Pita* genes in their genetic background. Genotypes Jirakatari, BR5, and Gopalbhog possessed *Pish* gene, while Uknimodhu, Deshikatari, Radhunipagol, Kalijira (3), Chinikanai each contained the *Pita* gene only. There are some materials that did not contain any target gene(s) in their genetic background, but proved resistant in pathogenicity tests. This information provided valuable genetic information for breeders to develop durable blast-resistant fragrant or aromatic rice varieties in Bangladesh.

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1. Introduction

Rice (*Oryza sativa* L.) confronts several major and minor diseases, among which blast caused by *Magnaporthe oryzae* [1] is a serious constraint causing moderate to huge economic losses worldwide [2–4]. Outbreaks of this

disease are a serious and recurrent problem, and are extremely difficult to control in all rice-growing regions of the world, including Bangladesh [5,6]. Bangladesh experienced several epidemic outbreaks of blast disease since 1980 [7], presumably because of the popularity of certain susceptible cultivars and/or generation of new pathogenic races. Most of the fragrant varieties are highly susceptible to this disease under favourable environment.

There are some chemicals available on the market to control this disease, but they are costly for resource-poor farmers, and sometimes effective chemical control is difficult in case of outbreaks, especially during neck blast.

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In addition, reduction of chemical application is also desirable for environmental protection in such a heavily farmed country as Bangladesh [8]. Cultivars resistance to blast is therefore the most eco-friendly and economic approach for managing blast diseases in rice. However, cultivars released as resistant often show high levels of susceptibility within a few years, even shortly after cultivar release, due to inadequate information of genotypes used as resistant sources and also due to a lack of information about the pathogen population structure. So, knowledge on population genetics of rice genotypes is important for breeding for blast disease resistance. Though cultivation of resistant varieties is the most promising method to control blast, the breeding-for-resistance programme is still at an early developmental stage in Bangladesh [9].

Fortunately, the Bangladesh Rice Research Institute (BRRI) has already collected more than 8000 rice germplasm. We believe that there are some resistant aromatic or fragrant sources in those materials that may contain blast-resistant gene(s) suitable for developing durable blast-resistant fragrant rice varieties. Recently, in collaboration with the Japan International Research Centre for Agricultural Sciences (JIRCAS), BRRI has developed good research facilities and started an international collaborative study under the “Blast Research Network for Stable Rice Production” project. In connection with this work, the present studies were undertaken with two major objectives: (1) to screen out the aromatic germplasm that are resistant against blast isolate and (2) to confirm the major blast-resistant genes in selected aromatic germplasm using tightly linked molecular markers for the development of durable blast-resistant rice varieties.

2. Materials and methods

2.1. Collection, isolation, purification, and preservation of blast isolate

Rice panicles of high yielding fragrant variety (BRRI dhan34) showing typical blast symptoms were collected from the rain-fed lowland ecosystem of Bangladesh. To obtain a single conidial isolate, an individual conidium was identified with a compound microscope (Olympus BX41) and aseptically transferred into a water agar medium. Finally, the fungus was grown on sterile filter paper and stored at -20°C after aseptic drying, necessary for repeated access to original isolates [10,11].

2.2. Inoculum preparation and inoculation

To produce inoculum, the stock isolate (paper disc) of single-spore culture was re-cultured in an oatmeal agar medium. The inoculated plates were incubated at temperatures from 25 to 28°C for 12 to 14 days. The culture was scraped with a sterilized tooth brush and the plates were exposed to continuous light for 4–5 days to induce heavy sporulation. Conidia were dislodged by gentle rubbing with a paintbrush from the incubated plates to sterilized distilled water with 0.01% Tween 20. Spore

suspensions were filtered through four layers of gauze mesh and concentration was adjusted to 10^5 conidia per millilitre using a haemocytometer.

Inoculation of 140 test materials, including 114 fragrant germplasm, 25 differential varieties (DVs) harbouring 23 blast-resistant genes [12–14] and LTH (universal susceptible check) was carried out following the standard methods [10,11]. The seeds of DVs were collected from JIRCAS, Tsukuba, Japan. Inoculated seedlings were incubated in a dew chamber at 25°C for 20 h, then transferred into a greenhouse maintained at $25 \pm 1^{\circ}\text{C}$ with 70 to 80% of relative humidity.

2.3. Disease assessment and data collection

Disease reactions of the inoculated plants were evaluated seven days after inoculation using a 0 to 5 scale, where 0 = no evidence of infection, 1 = brown specks smaller than 0.5 mm in diameter, no sporulation, 2 = brown specks about 0.5 to 1 mm in diameter, no sporulation, 3 = roundish to elliptical lesions about 1 to 3 mm in diameter with a grey centre surrounded by brown margins, lesions capable of sporulation, 4 = typical spindle-shaped blast lesions capable of sporulation, 3 mm or longer with necrotic grey centres and water-soaked or reddish brown margins, little or no coalescence of lesions, and 5 = lesions as in 4 but about half of one or two leaf blades killed by coalescence of lesions. The reactions of plant were further categorized as 0 to 2 as resistant (R), whereas 3 to 5 as susceptible (S) in most cases. The scores 2 to 5 were only categorized as susceptible for the differential variety of IRBL5-M and 4 to 5 for IRBLsh-B and IRBLta2-Pi [11,15]. The disease scoring data were then converted into the percent disease index (PDI) by using the following formula [16]:

$$\text{Percentdiseaseindex(PDI)} = \frac{\text{Sum of the scores}}{\text{Number of observation} \times \text{highest number in rating scale}} \times 100$$

Cluster analysis was done using Ward's hierarchical method using JMP 7.0.2 (JMP Statistics and Graphic Guide, Version 7.0.2: SAS Institute, Inc., Cary, NC, USA) based on a PDI of 140 tested materials. Canonical variant analysis was done using GenStat 5.3 software.

2.4. Confirmation of target genes in selected germplasm by molecular assay

2.4.1. Plant materials

Sixteen selected germplasm, four differential varieties (IRBLsh-B, IRBLta, IRBL9-W and IRBLta-2) that showed low PDI with blast isolate and one susceptible check (LTH) were used for the PCR assays. The plants were grown in a seedling tray for DNA isolation.

2.4.2. DNA extraction and PCR assay

DNA was isolated from leaves collected from 30-day-old seedlings as per the method of Murray and Thomson [17], with modifications. Polymerase chain reaction (PCR) was performed using gene-specific primers (Table 1). The

Table 1
Characteristics of primer used for blast resistant gene identification in selected germplasms.

Sl. No.	Target gene	Primar name	Size (base)	Primer sequence	References
1	<i>Pi9</i>	195R-1	20	ATGGTCCTTATCTTTATTG	[18]
		195F-1	19	TTGCTCCATCTCCTCTGTT	
2	<i>Pita-2</i>	RM155F	22	GAGATGGCCCCCTCCGTGATGG	[19]
		RM155R	22	TGCCCTCAATCGGCCACACCTC	
3	<i>Pish</i>	AOL51F	23	AGCTGCAGTAGTGCTGTCCATG	[20]
		AOL52R	20	CGTAAGATCATGAGCGAATG	
4	<i>Pita</i>	Pita403F	20	CAATGCCGAGTGTGCAAAGG	[21]
		Pita403R	20	TCAGGTTGAAGATGCATAGC	

primers of useful genes for Bangladesh conditions were selected from the reaction pattern of tested blast isolate with differential varieties. The PCR mix has a total volume of 10 μ L; it contains 25 ng of DNA template, 10 μ M of each primer, 25 mM of MgCl₂, 10 mM of dNTP, and 0.2 μ L of *Taq* polymerase. The PCR amplification conditions were 1 cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, at 55 °C for 45 s and 72 °C for 90 min, with a final extension at 72 °C for 10 min (G-Storm GS I thermal cycler). The PCR products were detected using 1.5% agarose gel electrophoresis and observation was recorded with a gel documentation system (Alpha Imager EP, Alpha Innotech, CA, USA). The PCR assays were repeated twice for confirmation purpose.

2.5. Yield and yield contributing characters of selected germplasms

The yield and yield-contributing characters of the selected germplasms were also evaluated at the Field of Plant Pathology Division, BRRI, Gazipur, Bangladesh during the rain-fed season (July–November 2012). Panicle length, filled–unfilled grain per panicle, and 1000 grain weight

were collected during harvesting. Principal component analysis was done using PAST multivariate software.

3. Results

3.1. Reaction of blast isolate to differential varieties

The percent disease indexes (PDI) of the tested blast isolate to 25 differential varieties (DVs) harbouring 23 resistant genes *Pish*, *Pib*, *Pit*, *Pia*, *Pii*, *Pi3*, *Pi5(t)*, *Pik-s*, *Pik-m*, *Pi1*, *Pik-h*, *Pik*, *Pik-p*, *Pi7(t)*, *Pi9*, *Piz*, *Piz-5*, *Piz-t*, *Pita-2*, *Pita*, *Pi12(t)*, *Pi19*, *Pi20(t)* and LTH are presented in Fig. 1. Among the tested DVs, twelve DVs having *Pia*, *Pii*, *Pi3*, *Pi5(t)*, *Pik-s*, *Pik-m*, *Pi1*, *Pik-h*, *Pik*, *Pik-p*, *Pi7(t)*, *Piz-t* genes, and LTH showed high PDI (> 75%), eight DVs having *Pib*, *Pit*, *Piz-5*, *Pi12(t)*, *Pita*, *Pi19*, *Piz* and *Pi20* genes showed intermediate reactions (50–75%), one DV having *Pish* gene showed PDI < 50%, and the remaining three DVs having *Pi9*, *Pita-2* and *Pita* genes showed lower (< 25%) PDI. DVs with low PDI showed four genes, *Pish*, *Pi9*, *Pita-2* and *Pita*, which might be the effective ones for developing a blast-resistant fragment rice variety.

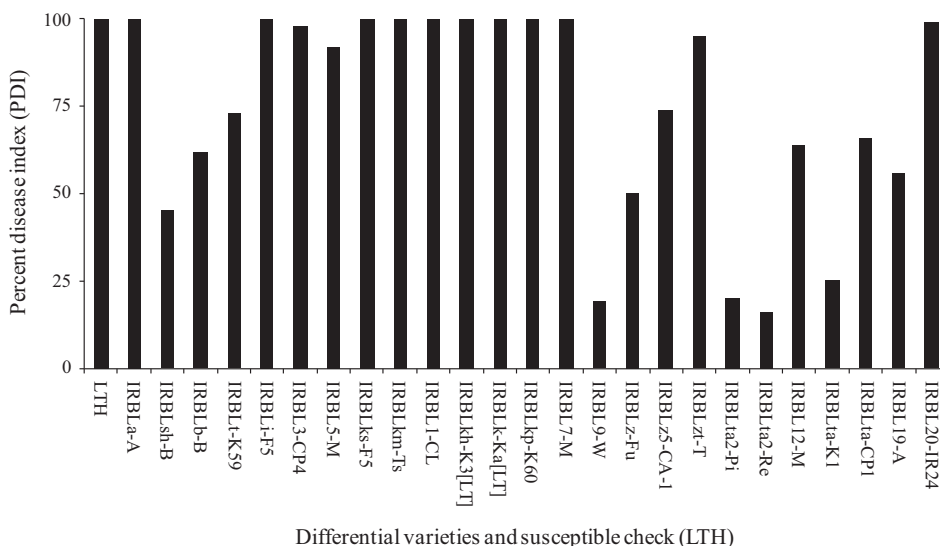


Fig. 1. Percent disease index (PDI) of tested blast isolate against differential varieties harboring 23 blast resistant genes and susceptible check (LTH).

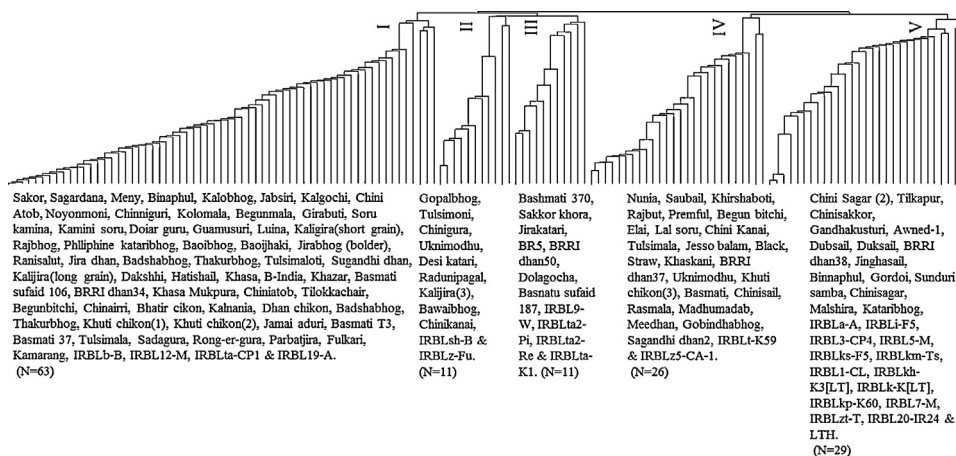


Fig. 2. Phenogram of 140 germplasm including fragrant, differential and susceptible varieties. The dendrogram was constructed depending on the reaction pattern of germplasm to blast isolate.

3.2. Clustering of genotypes

A dendrogram was constructed using a distance matrix which was calculated from percent disease index values (Supplementary Table S1). This dendrogram resolved the relationship among the 140 tested materials and grouped them into five clusters (Fig. 2). The distribution pattern indicated that cluster I was comprised of the highest number of tested entries (63), followed by cluster V (29), and cluster IV (26). Clusters II and III contained eleven entries each. Among the tested materials, susceptible check 'LTH' was placed in cluster V.

3.3. Canonical variant analysis (CVA)

Canonical Variant Analysis was performed to obtain the inter-cluster distances (Mahalanobis's D^2 value). The values of inter cluster distance (D^2) are presented in Table 2. Statistical distances represented the index of genetic diversity among the clusters. The inter-cluster distance was maximum between cluster III and cluster V (47.28), followed by the distance between cluster II and cluster V (34.57). The minimum inter-cluster distance was observed between cluster II and cluster III (11.17).

The mean PDI values of five clusters are presented in Fig. 3. The lowest mean value was found in cluster III, followed by cluster II. The genotypes under cluster II and III were resistant to blast disease. The highest mean value was found in cluster V, followed by clusters IV and I.

Table 2

Inter cluster distances (D^2) of 140 germplasm including aromatic, differential and susceptible varieties.

Cluster	I	II	III	IV	V
I	0.00				
II	11.53	0.00			
III	12.71	11.17	0.00		
IV	23.40	22.69	35.41	0.00	
V	23.88	34.57	47.28	11.87	0.00

3.4. Confirmation of useful genes in selected germplasm

Molecular analyses of the selected 16 germplasm of clusters II and III were done using a gene-based molecular marker along with resistant (target gene containing DVs) and susceptible material LTH. Germplasm of cluster II and III maintained the lowest cluster mean among the five clusters. The useful genes *Pish*, *Pi9*, *Pita-2* and *Pita* resistant against the Bangladeshi blast isolate from fragrant rice were determined by the reactions of the tested blast isolate with differential varieties (Fig. 1). The gel picture for the confirmation of *Pish*, *Pi9*, *Pita-2* and *Pita* genes are presented in Fig. 4. Among the selected germplasm, we found only three germplasm (BRRI dhan50, Chinigura, and Bawaibhog) that contained more than one target genes in their genetic background. Another eight germplasm contained only one target gene, either *Pish* or *Pita*. The rest of the materials did not contain any target gene(s) in their genetic background, though they showed resistant reaction in pathogenicity tests. None of the materials we found that contain *Pita-2* gene in their genetic background (Table 3).

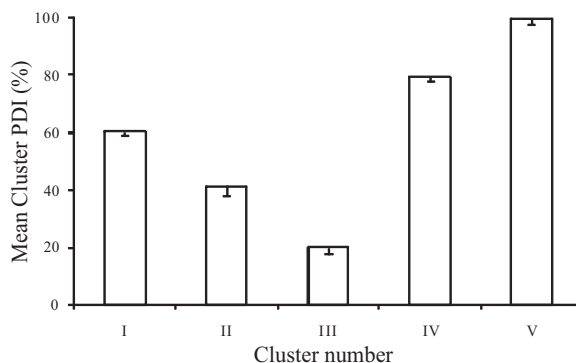


Fig. 3. Mean cluster percent disease index (PDI) of five clusters of 140 germplasm including fragrant, differential and susceptible varieties.

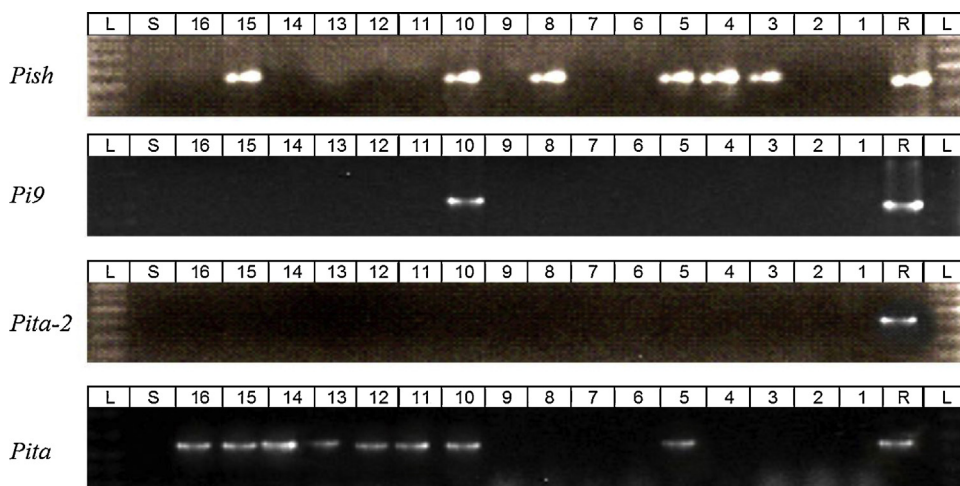


Fig. 4. (Colour online). PCR assay of the selected fragrant germplasms for different blast resistance genes (1 = Bashmati 370, 2 = Sakkorkhora, 3 = Jirakatari, 4 = BR5, 5 = BRRI dhan50, 6 = Dolagocha, 7 = Basnatusufaid 187, 8 = Gopalbhog, 9 = Tulsimoni, 10 = Chinigura, 11 = Uknimodhu, 12 = Deshikatari, 13 = Radhunipagol, 14 = Kalijira (3), 15 = Bawaibhog, 16 = Chinikanai, R = differential variety containing respective blast resistance single gene, S = susceptible variety LTH and L = ladder marker).

3.5. Yield potentiality of the selected blast-resistant land races

The results of yield potentiality of the selected resistant germplasms are presented in Table 4. Among these materials, Bashmati 370 provided the highest panicle length (27.7 cm). The sterility percentage of Bashmati 370 was also found in tolerable label (18.4%). The other variety, “Jirakatari”, was also found as long panicle (27.0 cm) and with the lowest sterility percentage (7.3%). The significant variations in grain size were found among these varieties.

Three principal components (PCs) accounted for 99.39% of the total variation in the 16 selected fragrant genotypes; of these, the first three PCs exhibited variations of 64.95, 33.82, and 0.61%. The character filled grain had the highest contribution, followed by unfilled grain (Fig. 5).

Table 3
Frequencies of useful blast resistant genes in selected germplasms.

Sl. No.	Designation of germplasms	BRRRI accession number	Presence of useful genes
1	Bashmati 370	4904	–
2	Sakkorkhora	5347	–
3	Jirakatari	5975	<i>Pish</i>
4	BR5	4343	<i>Pish</i>
5	BRRI dhan50	6882	<i>Pish</i> and <i>Pita</i>
6	Dolagocha	451	–
7	Basnatu sufaid 187	4499	–
8	Gopalbhog	672	<i>Pish</i>
9	Tulsimoni	1980	–
10	Chinigura	4867	<i>Pish</i> , <i>Pi9</i> and <i>Pita</i>
11	Uknimodhu	5083	<i>Pita</i>
12	Deshikatari	5978	<i>Pita</i>
13	Radhunipagol	6711	<i>Pita</i>
14	Kalijira (3)	247	<i>Pita</i>
15	Bawaibhog	301	<i>Pish</i> and <i>Pita</i>
16	Chinikanai	4356	<i>Pita</i>

BRRRI: Bangladesh Rice Research Institute.

– indicates resistant germplasm not containing any target genes in their genetic background.

4. Discussion

Significant variations were found among 140 materials in disease severity to blast, which indicated the existence of a genetic variability of blast resistance in the tested materials. This result was confirmed by the clustering pattern of the germplasms obtained through multivariate analyses. Sixteen fragrant germplasms such as Bashmati 370, Sakkorkhora, Jirakatari, BR5, BRRI dhan50, Dolagocha, Basnatu sufaid187, Gopalbhog, Tulsimoni, Chinigura, Uknimodhu, Deshi katari, Radhuni pagal, Kalijira(3), Bawaibhog, Chinikanai, and six differential varieties (DV) such as IRBL9-W, IRBLta2-Pi, IRBLta2-Re, IRBLta-K1, IRBLsh-B and IRBLz-Fu comprised in cluster II and III provided the lowest cluster mean of plant disease index (PDI). It indicated that materials of these clusters may contain blast-resistant useful genes *Pish*, *Pi9*, *Pita-2* and *Pita* in their genetic background, because DVs having these similar genes were in the same clusters (Fig. 2). The genotypes having lower PDI values under cluster II and III could be used as parents for the development of blast-resistant varieties. These results are in agreement with those of Latif et al. [22]. The other cluster (V) was comprised of 29 materials, including LTH maintained with the highest cluster mean PDI, which indicated that materials of this cluster are highly susceptible to blast (Fig. 3). The inter-cluster distance between II and III was minimum ($D^2 = 11.17$), indicating that the materials of these clusters were genetically closer. On the other hand, these two clusters maintained maximum distance from cluster V (Table 2).

Genotypes belonging to the distant clusters maintained maximum genetic variation [23]. This variability will greatly help researchers in selecting suitable donors in the breeding-for-resistance programme meant for transferring particular sets of genes into a desirable background [24]. Since the durability of single resistance genes to blast is very limited, efforts in the development of resistant

Table 4
Yield contributing characters of blast resistant selected fragrant germplasms.

Sl. No.	Designation of germplasms	Panicle length (cm)	Filled grain/panicle	Unfilled grain/panicle	Grain sterility (%)	1000 grain wt. (g)
1	Bashmati 370	27.7	110.7	20.3	18.4	18.7
2	Sakkorkhora	22.7	85.0	61.7	72.6	10.1
3	Jirakatari	27.0	123.0	9.0	7.3	16.0
4	BR5	22.0	221.7	105.0	47.4	16.1
5	BRRI dhan50	23.3	122.3	36.7	30.0	12.3
6	Dolagocha	25.7	126.3	48.7	38.5	11.1
7	Basnatusufaid 187	25.3	114.7	76.0	66.3	16.6
8	Gopalbhog	25.5	115.0	75.5	65.7	11.6
9	Tulsimoni	26.0	170.0	72.0	42.4	7.5
10	Chinigura	23.7	153.3	17.7	11.5	23.1
11	Uknimodhu	23.0	118.7	61.7	52.0	9.3
12	Deshikatari	23.7	107.7	58.0	53.9	8.2
13	Radhunipagol	21.3	107.7	37.0	34.4	11.0
14	Kalijira (3)	26.7	173.7	37.0	21.3	7.6
15	Bawaibhog	24.3	232.3	51.3	22.1	13.9
16	Chinikanai	23.7	116.3	24.0	20.6	10.6

varieties are geared towards stacking of genes to develop gene pyramids. For sustainable utilization of such gene pool for providing an assured resistance against blast, a comprehensive screening of all these accessions against different pathotypes to assess the expression levels of the genes detected is an essential first step for confirmation of useful donors. As expected, the *Pita-2* gene was not detected in these accessions; therefore, more rice entries need to be screened to detect this gene. In addition to providing sources of resistance, these germplasms can be extremely useful as the experimental material to develop blast-resistant fragrant rice varieties. Researchers from

Japan [25], China [26], India [27], Philippines [28], Thailand [29], and Bhutan [30] screened large number of genotypes and found rice genotypes with resistant genes against blast pathogens. In Bangladesh also, huge numbers of rice entries were screened against blast and resistant entries were identified [31,32]. However, no attempt has so far been made to identify the gene involved, which is essential for maintaining and utilizing the known gene pool.

The results of pathogenicity tests provided important information for selecting useful genes to develop blast-resistant fragrant rice varieties and gene-based markers for molecular study. From the reaction between blast isolate

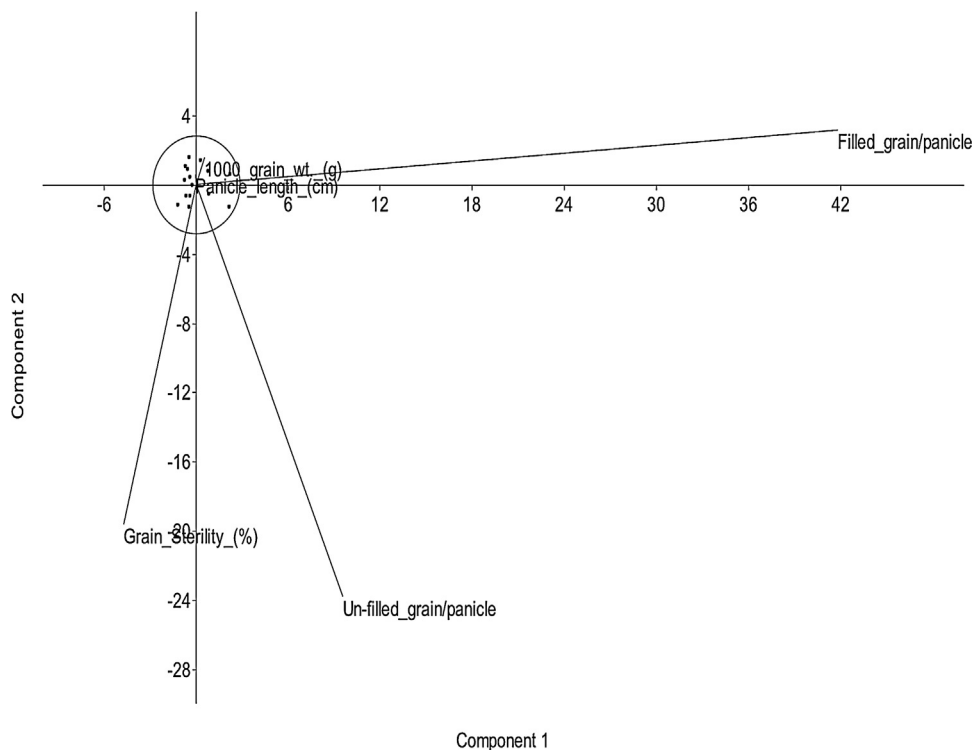


Fig. 5. Principal component analysis of 16 selected germplasms based on 5 yield attributing characters.

and differential varieties, we found *Pish*, *Pi9*, *Pita-2* and *Pita* as being effective against blast isolate. Based on this result, tightly linked gene based marker AOL51 and AOL52 for *Pish*, RM195 for *Pi9*, RM155 for *Pita-2* and Pita403 for *Pita* were selected for molecular analyses. Koide et al. [25] carried out a detailed study on the resistance genes and selection DNA markers for blast disease in rice. They found effective markers that were tightly linked to the blast-resistant genes.

5. Conclusion

To identify blast-resistant fragrant sources, the combination of DNA markers and pathogenicity assays is the best way to select donors for the development of durable blast-resistant rice varieties. In this study, only the rice cultivar “Chinigura” contained blast-resistant genes *Pish*, *Pi9* and *Pita*, while both BRRI dhan50 and Bawaibhog contained *Pish* and *Pita* genes in their genetic background. This material may be used as a gene pool to improve blast resistance in rice using marker-assisted or conventional breeding.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.crv.2014.02.007>.

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