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Identification of Broad Spectrum Blast Resistance Genes for North-East and Eastern India using Standard International Blast Differential

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A B S T R A C T

Rice Blast caused by the fungal pathogen *Magnaporthe oryzae* is one of the most devastating diseases worldwide. Host plant resistance is the effective and economical way to manage this disease. Host plant resistance has been exploited as source for developing resistant variety since past. Challenge (host resistance) induced shifts in pathogen variabilities necessitates continuous development of need based resistant varieties. Knowledge on ever-changing and location specific variability pattern of pathogen, with reference to known, available blast resistant genes, is prerequisite for such efforts. Attempt was made in the present study to analyze virulence spectrum of 90 *M. oryzae* isolates collected from different geographical regions of North-East and Eastern India using monogenic differentials targeting 26 major blast resistant genes *Pi9*, *Piz5(Pi-2)*, *Pita*^{2}, *Pita*^{2}, *Piz*, *Pi1*, *Pi5*, *Pi7*, *Pii*, *Pi20(t)*, *Pi11*, *Pi-kh*, *Pi-km*, *Pi-ks*, *Pi12(t)*, *Piz-t*, *Pi-sh*, *Pik*, *Pib*, *Pi3*, *Pit*, *Pi19(t)*, *Pita*, *Pi-kp*, *Pita (Pi-4)* and *Pi-a* under green house conditions. Resistance percentage ranged from 19.7% to 94.2 % among the monogenic lines. All the 90 isolates produced virulent reaction on susceptible check Lijiangxintuanheigu (LTH). *Pi9*, *Piz5(Pi2)*, *Pita*^{2}, *Piz and Pi1* genes showed wide resistance spectra respectively and can be important R gene for preventing blast disease. Matching virulence to all resistance genes were detected in the pathogen population. The genes *Pi9* (94.2%) and *Pita*^{2} (78.2%) showed complementary resistance spectrum and the monogenic lines carrying these genes together, showed resistant reaction to all 90 isolates. These results suggest that combination of *Pi-9 + Pita*^{2}, *Pi9 + Piz5*, *Pi9 + Piz*, *Pi9 + Pi1*, *Piz5 + Pi1 and Piz + Pi1* may play an important role in prevention of blast disease across all the locations. Based on above data, a useful strategy can be formulated for the management of rice blast disease by stacking R-genes against pathogenic *M. oryzae* isolates for this geographical region.

Introduction

Blast disease of rice caused by filamentous fungus *Magnaporthe oryzae* (Couch and Kohn, 2002), is a devastating disease affecting rice production every year globally (Ou, 1985). The pathogen infects rice crop at every growth stages starting from seedling to grain filling stage and cause substantial yield loss (Dean *et al.*, 2005). India is second

largest rice producing nation of the world. In India, rice blast is prevalent in all rice growing regions with highest incidence in Eastern India followed by North and South India (Variar *et al.,* 2009; Khush and Jena, 2009). According to one estimate, in Eastern India about 564,000 tons of rice is lost due to blast of which nearly 50% (246,000 tons) is in the upland ecosystem (Widawsky *et al.,* 1990). Blast fungus shows a high degree of variability in the field leading to frequent emergence of new races knocking down prevalent resistant cultivars (Valent *et al.,* 1991). Any change in frequency of virulence directly challenges the effectiveness of resistant cultivars (Kiyosawa., 1982). Further, transposable elements (TE) have been implicated in the emergence of virulent forms of the pathogen by their frequent insertion into avirulence genes as reported in case of *Avr-Pita, Avr-Piz-t* and *Ace1* gene (Zhou *et al.,* 2007; Li *et al.,* 2009 and Bohnert *et al.,* 2004). Presence of TEs in *M. oryzae* population poses a continuous threat to the effectiveness of existing blast resistant cultivars. The average life span of many resistant rice cultivars is 2 to 3 years in blast prone environment (Leung *et al.,* 1988). This necessitates continuous, location specific monitoring of pathogen variability based on virulence analysis against known major blast resistance genes for selection and deployment of effective genes or their combinations against prevalent pathotypes and maintain regular release of resistant cultivars in certain interval. Virulence analysis or pathotyping is a vital tool to determine the race variation, pathotype composition and effective blast resistance genes for any geographical location. This analysis is done with a genetically well-defined set of resistant sources (differentials) to obtain high degree of resolution for describing the virulence structure of a population. A new set of 26 differential varieties targeting 24 resistance genes in the genetic background of Lijinanxintuanheigu (LTH) developed at International Rice Research Institute (IRRI) in collaboration with Japan International Rice Research Center for Agricultural Sciences (JIRCAS) (Tsunematsu *et al.,* 2000; Kobayashi *et al.,* 2007) was used to understand pathogenic variability in *M. oryzae* (Fukuta *et al.,* 2010).

Blast disease can be controlled by various fungicides which are not environmentally safe and continuous use poses threat to emergence of resistant pathogen races (Kim *et al.,* 2008). Use of resistant cultivar integrated with cultural practices is the most economical and effective way to control this disease (Roy Chowdhury *et al.,* 2012; Bonman, 1992 and Lee, 1994). Several blast resistant varieties have been released but their resistance was knocked down few years after release because of emergence of novel pathogenic variation. Transposable elements have been implicated in the emergence of virulent forms of the pathogen by their frequent insertion into avirulence genes as reported in case of *Avr-Pita, Avr-Pizt* and *Ace1* gene (Zhou *et al.,* 2007; Li *et al.,* 2009 and Bohnert *et al.,* 2004). Presence of TEs in *M. oryzae* population poses a continuous threat to the effectiveness of existing blast resistant varieties. Therefore, virulence analysis is prerequisite to determine the diversity in the pathogen population and selection of effective blast resistant genes or their combination for the management of this disease.

In our previous study on molecular diversity and mating type distribution of *M. oryzae* isolates from North-East and Eastern India by *Pot2*-TIR and MGR586-TIR clearly indicated that high lineage diversity exist in this region. Eight and nine lineages from two different primers were identified in this region. Presence of both the mating type in the isolates of this region suggested the possibility of sexual recombination in nature

which can affect the diversity and dissemination (Imam *et al.,* 2015). Present study was taken up to determine the virulence diversity of *M. oryzae* isolates of this geographical region. Forty-three isolates representing each lineage and another forty-seven new isolates were selected for this study. The main objective of the present investigation is to (1) determine the virulence diversity of *M. oryzae* isolates (2) identify the effective resistance genes for this region and (3) develop breeding strategies for stacking multiple *R* genes for durable blast resistance in North-East and Eastern India.

Materials and Methods

Collection and maintenance of isolates

The present study was conducted on *M. oryzae* isolates of Eastern and north eastern part of India collected from Assam, Jharkhand, Odisha, Meghalaya and Tripura over a period five years (2010-15) (Table 1). Two hundred- fifty blast infected leaf and neck blast samples were collected from different part of North-East and Eastern India during 2010 to 2015. Most of the collections were made from farmer's fields. Leaf blades with necrotic lesions were washed in tap water for 1 to 3 min and surface sterilized with 0.1% mercuric chloride. They were then washed serially with double distilled water and allowed for sporulation on sterilized glass slide by incubating in moist chamber at 28°C for 24 h. Conidia were dislodged from individual sporulating lesions onto 2% agar plates with a sterilized glass needle. Single spores were picked up aseptically under a microscope and transferred to fresh Oat Meal agar slant. From each leaf or neck samples, mono-conidial isolates were prepared and maintained on desiccated filter paper following the procedure described by Hayashi *et al.,* 2009. A total of ninety single spore isolates were selected on the basis of their sporulation ability for virulence analysis (Table 1).

Monogenic differentials for virulence analysis

Twenty-six international differentials (Tsunematsu *et al.,* 2000; Kobayashi *et al.,* 2007), each having single blast resistance gene introgressed into LTH genetic background was used in this study. The rice variety LTH was used as susceptible check and Tetep as resistant check. Five to seven seeds of each differential variety were planted in plastic trays (54 x 36 x 7 cm) filled with mixture of soil and FYM (3:1). All the trays were kept in green house at 25±1^{o}C for 21 days until fourth leaf half emerged.

Inoculation and disease evaluation

Inoculation of blast isolates was done following the method of Hayashi *et al.,* (2009) with slight modification. The stored cultures colonized on desiccated filter paper were grown on oat meal agar slants. Mycelia from 10-day-old slants were macerated in 5 ml of distilled water and plated onto OMA plates. The plates were incubated at 25$\pm$1^{o} C for 6 days under fluorescent light for sporulation. After sporulation 10 ml of sterilized distilled water was poured on to each culture plate. Using sterilized glass slide, the fungal growth surface was scraped and filtered through two layers of sterilized cheese-cloth. The spore concentration was adjusted to 1 x 10^{5} spore/ml. Tween-20 (@ 0.01%) was added to the spore suspension as adhesive. The inoculum was sprayed onto 21 day- old seedlings using fine sprayer. The inoculated plants were then transferred to humidity chamber for 24 hours after which they were incubated in the greenhouse at 25$\pm$1^{o} C for 6 days. Disease reaction of each differential line was evaluated 7 days after inoculation on 0-5 scale according to the

Standard evaluation system (SES, 1996) for rice blast developed at IRRI. The reactions of differential varieties were categorized into three classes viz.; 0-3= resistant (R), and 4-5= susceptible (S) (Zhou *et al.,* 2003).

Data analysis

Resistance percentage and virulence frequency were calculated according to the following formulas:

Resistance percentage (%)= number of incompatible isolates / total number of rice lines or R genes tested ×100

Virulence frequency (%)= number of virulent isolate on R genes/ total number of isolate tested ×100 (Saad *et al.,* 2010)

Results and Discussion

Useful blast R-genes

Disease reaction of 90 *M. oryzae* isolate of North-East and Eastern India against twenty-six international differentials revealed that *Pi9, Piz5(Pi2), Pita*^{2}*, Pi1 and Piz* are potential resistance gene for resistance breeding program as they exhibited compatibility with less number of isolates. The percentage of resistance on international differentials targeting 26 major R-genes viz., *Pi9, Piz5(Pi-2), Pita*^{2}*, Pita*^{2}*, Piz, Pi1, Pi5, Pi7, Pii, Pi20(t), Pi11, Pi-kh, Pi-km, Pi-ks, Pi12(t), Piz-t, Pi-sh, Pik, Pib, Pi3, Pit, Pi19(t), Pita, Pi-kp, Pita (Pi-4)* and *Pi-a* ranged from 19.7% to 94.2 %. The resistance check Tetep was found resistant to all isolates. Tetep is known to harbor at least four major blast resistance genes *Pi1, Pita, Pit* and *Pi54* (Inuikai *et al.,* 1995) which contribute to its broad spectrum resistance. The percentages of resistant on monogenic lines carrying *Pi9, Piz5(Pi2), Pita*^{2}*, Piz and Pi1* were 94.2%, 92.5%, 78.2%, 71.5% and 67% which showed that these genes have wide resistance spectra to the prevalent isolates and can be useful to prevent blast disease in this region (Fig. 1 and Table 2).

Virulence spectrum of M. oryzae isolates

The study revealed that high virulence diversity exists in pathogen population of North-East and Eastern India (Table 3). All the Isolates were found virulent to one or more monogenic lines. The pathogen population comprises isolates virulent to minimum 3 to maximum 22 resistant genes out of 26. All isolates were virulent to LTH. Virulence frequency of different *M. oryzae* isolates was found to range from 10.7% (Mo-ei-163) to 78.5% (Mo-ei-5a) (Fig. 2). Isolates originating from Jharkhand were more virulent than the isolates from other region of North-East and Eastern India as they exhibited compatibility with large number of resistant genes. Out of 90 isolates, Mo-5, Mo-ei-76, Mo-ei-43 and Mo-ei-103 originating from Jharkhand had the highest virulence (Fig. 2).

Gene combination strategy to develop durable resistance

Matching virulence to all resistance genes were detected in the pathogen population. Most of the blast resistant genes expressed narrow resistant spectrum except few (*Pi9, Piz5(Pi2), Pita*^{2}*, Piz and Pi1)* to the tested blast isolates of Eastern India, suggesting that most resistance genes were effective against a part of the pathogen population and it would be impossible to obtain desirable levels of resistance by the introgression of single resistant gene. Therefore, pyramiding of two or more resistant gene showing complementary resistant spectra will be needed to develop durable resistance. As shown in Table 3 and Figure 1, *Pi9* gene expressed high level of resistance to most of

the isolates (94.25%) and can be exploited in combination with other effective resistant genes for achieving broad spectrum resistance. To excluding all the virulence of the pathogen population, various combinations of *Pi9* and its allelic genes *Piz, Piz5(Pi2)* 1) *Pi9 + Pita2;* 2) *Pi9 + Piz5;* 3) *Pi9 + Piz;* 4) *Pi9 + Pi1;* 5) *Piz5 + Pi1; and* 6) *Piz + Pi1* were constructed. Out of all the combination, *Pi9 + Pita*^{2} are expected to provide broad spectrum resistance across all the location by excluding all virulences of pathogen population.

North-East and Eastern region of India is considered as one of the hot pocket of rice genetic resource with extremely diverse rice growing conditions as compared to other parts of the country. Rice blast is endemic and major disease of this region because of high humidity during growth stage of rice. Until recently, pathogen variability was being studied as response to a set of differentials (Ling and Ou, 1969; Atkins *et al.,* 1976, Yamada *et al.,* 1976; Kiyosawa *et al.,* 1984). However, the older differential varieties were inadequate to describe the genetic and phenotypic variability of *M. oryzae* populations because they were not uniform, contain more than one gene, also not present in single genetic background. The present study demonstrated that new monogenic differentials targeting 26 resistance genes (Tsunematsu *et al.,* 2000; Kobayashi *et al.,* 2007) in blast susceptible recurrent parent LTH are excellent material to identify the resistance spectra of resistant genes precisely against the blast isolates tested from North-east and Eastern India. In our previous study on molecular diversity and mating type distribution of 63 *M. oryzae* isolates from North-East and Eastern India by *Pot2*-TIR and MGR586-TIR clearly indicated that high lineage diversity exist in this region. Eight and nine lineages from two different primers were identified in this region (Imam *et al.,* 2015). Present study demonstrated virulence diversity of *M. oryzae* isolates of this geographical region. Based on our finding we proposed a strategy for stacking blast R-genes to achieve longer lasting resistance. Our results suggested that *Pi-9, Piz5(Pi2), Pita*^{2}*, Piz and Pi1* are most effective resistance genes and recommended to rice breeders for improving blast resistance in this region.

Table.1 Number of blast isolate phenotyped from different sites of Eastern and north eastern India

Sl.No.	State	District	No. of isolates Phenotyped
1	**Assam**	Gerua	10 isolates
		Titabor	4 isolates
		Jorhat	1 isolate
2	**Tripura**	Lembucherra	6 isolates
3	**Meghalaya**	Barapani	1 isolate
4	**Jharkhand**	Ranchi	8 isolates
		Hazaribag	35 isolates
		Itkhori	1 isolate
		Sankarpur	3 isolates
		CRURRS, farm	17 isolates
5	**Orissa**	Semiliguda	4 isolates
		Total	**90 isolates**

Table.2

Sl.no.	Designation	R-Gene	No. of incompatible isolate	Percentage of Incompatible isolate (%)
1	IRBL1-CL	*Pi1*	59	67.04
2	IRBL11-ZH	*Pi11*	47	53.4
3	IRBL12-M	*Pi12(t)*	47	52.22
4	IRBL19-A	*Pi19(t)*	22	24.44
5	IRBL20-IR24	*Pi 20(t)*	39	46.98
6	IRBL3-CP4	*Pi3*	30	33.7
7	IRBL5-M	*Pi5*	56	62.92
8	IRBL7-M	*Pi7*	20	35.08
9	IRBL9-W	*Pi9*	82	94.25
10	IRBLA-A	*Pia*	24	26.66
11	IRBLB-B	*Pib*	33	37.07
12	IRBLI-F5	*Pii*	47	52.8
13	IRBLK-KA	*Pik*	33	37.07
14	IRBLKH-K3	*Pikh*	42	46.66
15	IRBLKM-TS	*Pikm*	43	47.77
16	IRBLKP-K60	*Pikp*	26	28.88
17	IRBLKS-F5	*PikS*	46	51.11
18	IRBLSH-S	*Pish*	40	44.44
19	IRBLT-K59	*Pit*	29	32.95
20	IRBLTA-CT2	*Pita (Pi4)*	16	19.75
21	IRBLTA-K1	*Pita*	27	30
22	IRBLTA2-PI	*Pita2*	68	78.16
23	IRBLTA2-RE	*Pita2*	61	78.2
24	IRBLZ-FU	*Piz*	63	71.59
25	IRBLZ5-CA	*Piz5 (Pi2)*	75	92.59
26	IRBLZT-T	*Pizt*	38	42.22
27	Tetep	Tetep	89	100
28	LTH	LTH	0	0

Fig.1 Resistance spectra of different R- genes

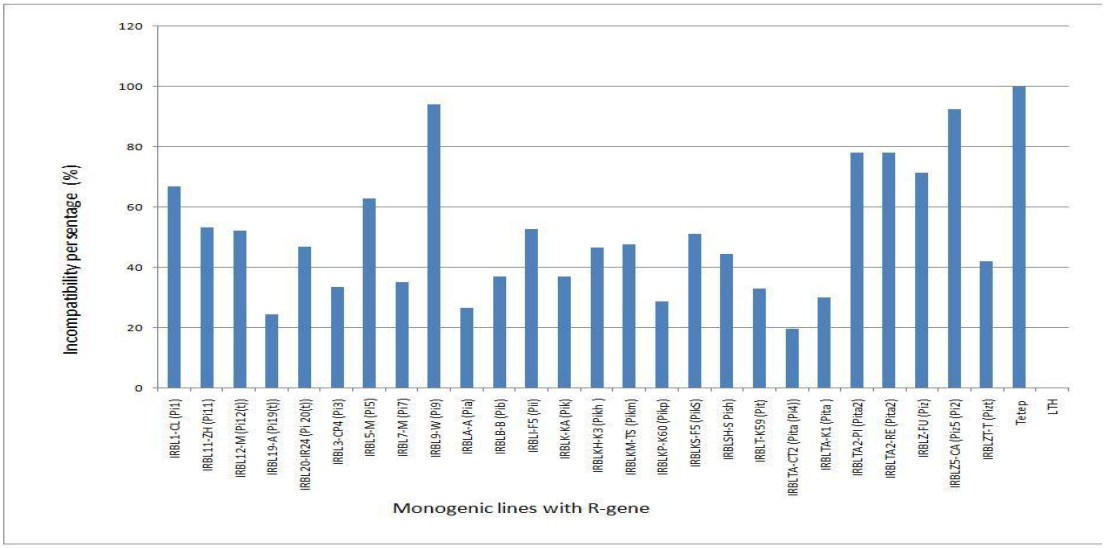

Fig.2 Virulence frequency of *M. oryzae* isolates (%), AS: Assam, Jh: Jharkhand, MG: Meghalaya, OR: Odissa, TR: Tripura

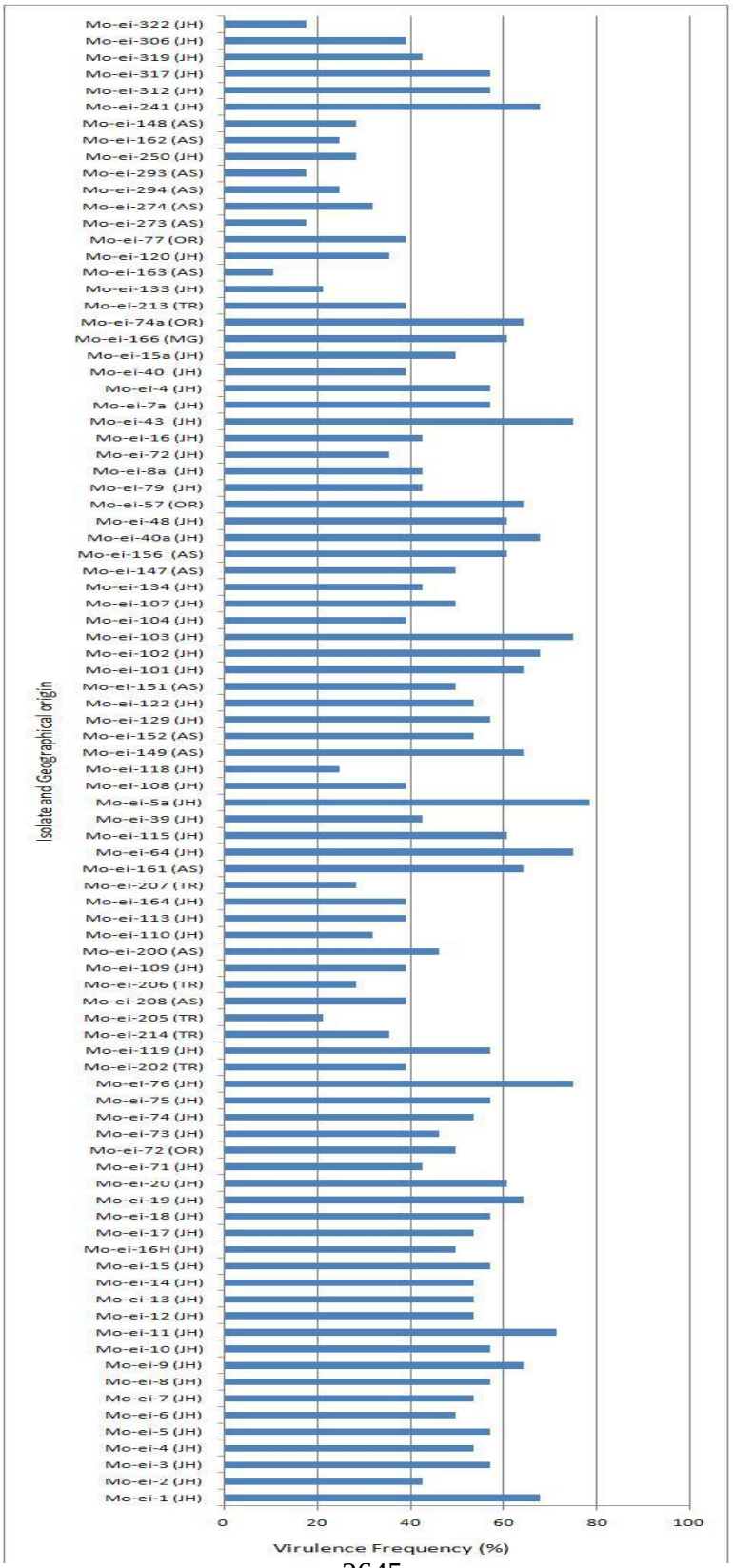

Table 3: Effective gene combinations for deployment in different Eastern and North Eastern states of India *IRBLTA2-RE

Sl.no.	Gene combinations	Percentage of pathogen population excluded				
		Jharkhand	Orissa	Assam	Meghalaya	Tripura
1	*Pi9 + Pita2**	100	100	100	100	100
2	*Pi9 + Piz5*	98.18	100	100	100	100
3	*Pi9 + Piz*	96.36	100	93.3	100	100
4	*Pi9 + Pi1*	96.36	100	100	100	100
5	*Piz5 + Pi1*	96.36	100	87.5	100	80
6	*Piz + Pi1*	90.9	100	75	100	100

However, virulence to almost all the resistant genes was identified in pathogen population. None of the genes showed incompatible reaction to all the isolates except Tetep. Therefore, stacking of multiple R-genes in combination is an excellent strategy to achieve broad spectrum resistance. Results suggest that among the R-gene combinations identified, *Pi-9 + Pita*^{2} appear to have potential for effective management of rice blast disease across all locations by excluding all virulences of North-East and Eastern Indian pathogen. DNA markers closely linked to major blast resistance genes are available and their incorporation can now be accelerated using marker assisted selection. To our knowledge, this was the first report to describe the resistance spectra of 26 different blast resistant genes providing information about possible combination of genes which could be used to develop durable system of protection to this blast disease in this region.

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Abbreviation: *M. oryzae*: *Magnaporthe oryzae*; LTH: Lijinanxintuanheigu; *Avr*: Avirulence; IRRI: International Rice Research Institute; SES: Standard evaluation system*;* R: Resistant; S: susceptible; R- gene: Resistant gene*;* AS: Assam; Jh: Jharkhand; MG: Meghalaya; OR: Odissa; TR: Tripura

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