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Pathological and molecular characterizations of slow leaf rusting in fifteen wheat (*Triticum aestivum* L. em Thell) genotypes

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Leaf rust caused by *Puccinia triticina*, is a globally important fungal disease of wheat (*Triticum aestivum* L. em. Thell), resulting in significant yield losses, sometimes up to 40% worldwide. In this study we investigated slow rusting resistance at pathological and molecular level. Fifteen (15) wheat genotypes which also included multiple crosses with the aim to characterize pyramid resistance genes, including slow rusting genes like *Lr46* and *Lr50* were evaluated for disease severity percent, latent period and incubation period under field conditions. Detached leaf assay was also performed with three virulent pathotypes *viz.,* 21R55 (104-2), 121R63-1 (77-5) and 29R45 (12-5), under controlled laboratory conditions. Genotypes, KIRITATAI//HUW234+LR34/PRINIA, WAXWING*2/TUKURU, WBLLI*2/KIRITATI, KAMBI*2/-BRAMBLING and KAMBI*2/KIRITATI were very close to near immunity and showed comparatively higher level of resistance against all the three pathotypes. Disease severity in resistant genotypes was traced type 5 to 6% in both years, while it was 60 to 80% in the case of susceptible genotypes, that is, 'Agra Local' (S1). Similar pattern was observed for AUDPC, that is, <250.0 in the resistant genotypes, while it was beyond 1000.0 in 'Agra Local'. The shorter mean latent (7.67) and incubation period (6.0) was observed in susceptible genotypes, that is, 'Agra Local' to all the resistant genotypes, that is, LP (10 to 12) and IP (9 to 10); while testing against all the three different pathotypes. Linked microsatellite markers were used to confirm the presence of different rust resistance genes required to achieve near immunity. Out of 10 primers, nine produced gene specific bands with all genotypes except the control, that is, Agra Local. Genotypes which showed slow rusting, had longer latent period and incubation period as well as reduced percent disease severity and confirmed the presence of four to five resistance genes including slow rusting genes, that is, *Lr46* and *Lr50.* This indicates that these genotypes have potential durable resistance and can be used as parental lines in the development of more durable rust resistance.

Key words: Near immunity, pathotypes, *Puccinia triticina*, SSR.

INTRODUCTION

Leaf rust of wheat caused by *Puccinia triticina* Ericks., previously known as *Puccinia recondite* f. sp. *tritici,* is one of the most damaging diseases and therefore, is an important disease of wheat in many parts of the world. The average yield losses due to leaf rust (brown rust) are reported to vary between 15 to 60% (Mcintosh, 1998).

Losses caused by leaf rust in susceptible varieties have exceeded 50% in some years. In favorable environmental conditions, if not controlled in irrigated fields, leaf rust may cause severe losses almost every year and causes up to 60% of yield loss. During the last three decades, there has been a remarkable progress in breeding for development of rust resistant varieties, which in turn prevented the calamity of rust epidemic in different epidemiological sub-zones of the country and reduced the competition among pathogens (Bahadur et al., 1994; Singh et al., 2011). Despite of all these advancements, leaf rust is still considered as major constraint limiting successful production of wheat worldwide (Singh et al., 2011).

Leaf rust is an air-borne disease and due to yearly changes in weather, acreage planted to susceptible varieties and frequent development of new races of the fungus that are capable of attacking varieties previously resistant. Occurrence of leaf rust is unpredictable. Wheat cultivars have not shown durable resistance against *P. triticina*, though scientist at CIMMYT, Mexico and other places are trying hard to bring durable resistance for leaf rust. Durable resistance may be controlled by a single gene, multiple genes with cumulative effect or polygenes and the resistance produced may be either complete or incomplete (partial). A total of 67 genes conferring resistance to leaf rust have been catalogued till date (http://wheat.pw.usda.gov/GG2/index.shtml). These genes alone or in combination provide a satisfactory level of resistance. A number of genes such as *Lr9, Lr19* and *Lr24*, are effective against most of the pathotypes of leaf rust, and are available in the improved genotypes, but sometimes, these resistant genes lack durability. Thus, the short lived nature of race-specific hypersensitive response has created the necessity to search for more durable type of resistance. The most durable and best strategy for the control of rusts, lies in combining genes irrespective of whether the genes are minor or major (Sawhney, 1995). In recent years, increased awareness of the short lived nature of the vertical resistance to wheat rust has led to more emphasis on the phenomenon of general resistance called slow rusting which has been reported in rust fungi (Shaner et al., 1978; Sharma et al., 1996). Several workers reported that some varieties had the ability to retard rust development even though they had a susceptible reaction type (Caldwell et al., 1970; Singh et al., 1991). Slow rusting of cultivars has attracted the attention of breeders and pathologists. The components that cause slow rusting of a cultivar are longer disease latent period, low receptivity or infection frequency, as well as smaller uredial size,

reduced duration and quantity of spore production. All these components can affect disease progress in the field (Wilcoxson, 1981; Hartleb et al., 1984; Navi et al., 1989).

Slow rusting is a useful measure of resistance because it is the result of all factors that influence disease development such as differences in environment, cultivars and population of the pathogen. Germplasm showing slow rusting have been found useful in the development of more durable leaf rust resistant wheat cultivars (Singh et al., 2000). The wheat varieties available for commercial cultivation do carry some known disease resistance genes against rusts but these known resistance gene(s) are unable to provide durable resistance to the wheat cultivars. In the sustainable agriculture, which is economical both for the farmer and nature, durable disease resistance is an essential tool against pathogens attack beside cultural practices, like crop rotation, seed treatment etc. Moreover, with the biotrophic fungi like rusts and powdery mildew, the only solution is the durable disease resistance (Nagarajan et al., 1998). So far, a number of leaf rust resistance genes are known to provide complete protection (Mcintosh et al., 1995) and the combination of minor genes with major disease resistance genes have been found to be an effective strategy for attaining durable resistance (Singh et al., 1995). However, till date, none of the available variety is completely immune against leaf rust pathogens. Slow rusting has been characterized separately at pathological and molecular level, but so far, not a single effort has been made to combine both the approaches together. Therefore, efforts were made to have a comprehensive evaluation of wheat genotypes following a combined approach of pathological and molecular approaches.

In the last two decades, advances in the field of molecular markers have contributed towards identification of genes/quantitative trait loci (QTLs) for the various leaf rust resistant genes (Mcintosh et al., 2011). Though, various molecular markers are available for plant genotyping, simple sequence repeats (SSRs) have found large scale application in mapping of genes due to several advantages such as they are highly polymorphic, highly reproducible and uniform distribution throughout the genome in comparison to other markers.

Now, a number of SSR markers has shown their linkage with various leaf rust resistance genes which are currently being used in marker assisted selection (Gultyaeva et al., 2009). Despite the importance of slow rusting mechanism in the control of rust epidemics, very few efforts has been made to combine the information of differential reaction with various rust isolates and molecular markers linked with slow rust resistant genes, which can play a vital role in the development of more resistant cultivars against leaf rust. Impact of slow rust resistant genes alone or in combination to achieve durable resistance/near immunity is yet to be done. Genes responsible for slow rusting can be brought

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Abbreviation: AUDPC, Area under disease progress curve.

Table 1. The details of all the fifteen genotypes used in the present study.

S/N	Genotype (cross)	Denoted by
1.	HUW 234 [HUW 12*/CPAN 1966 (Sparrow)]	G-1
2.	KIRITATI/4/SERI.1B*2/3/KAUZ*2/BOW//KAUZ	G-2
3.	KIRITATAI//PRL/*PASTOR	G-3
4.	KIRITATAI//ATTILA*2/PASTOR	G-4
5.	KIRITATAI//HUW234+LR34/PRINIA	G-5
6.	KIRITATAI//WBLLI	G-6
7.	WEAVER/TSC//WEAVER/3/WEAVER/4/PRL/2*PASTOR	G-7
8.	PFAU/SERI.1B//AMAD/3/WAXWING	G-8
9.	WAXWING*2/VIVITSI	G-9
10.	WAXWING*2/TUKURU	G-10
11.	WBLLI*2/KIRITATI	G-11
12.	KAMBI*2/BRAMBLING	G-12
13.	KAMBI*2/KIRITATI	G-13
14.	HUW468 [CPAN 1962/TONI//LIRA/PARULA]	G-14
15.	Agra Local	S-1

together with the help of molecular markers. Once molecular markers linked to the gene of interest are known, it is easy to combine multiple resistant genes in a recipient genotype using marker assisted selection. The increasing threat of leaf rust, calls for serious efforts to understand various dimensions of slow rusting to breed resistant genotypes. In view of the importance of the leaf rust disease of wheat, the present study was designed to generate information about the impact of slow rusting resistance genes, differential reaction of rust isolates and molecular characterization of wheat genotypes for the presence of slow rusting genes with the help of associated SSR markers. The other purpose was to characterize the effect of slow rusting genes on the disease severity. The 12 CIMMYT wheat genotypes used in present study were developed by multiple crosses with the aim to achieve the near immunity through pyramiding the resistance genes including slow rusting genes like *Lr46* and *Lr50*. Two elite Indian wheat resistant cultivars, that is, HUW234 and HUW468 and one known leaf rust susceptible cultivar, that is, Agra Local were included as control.

MATERIALS AND METHODS

Plant material and leaf rust pathotypes

Field trials were conducted during 2006-07 and 2007-08 Rabi seasons at the Crop Research Centre (CRC), Sardar Vallabh Bhai Patel University of Agriculture and Technology, Meerut, U.P., India. 15 wheat genotypes including 12 CIMMYT entries developed by multiple crosses between different leaf rust resistant lines with the aim to achieve the near immunity through pyramiding the resistance genes including slow rusting genes like *Lr46* and *Lr50*. Two elite Indian wheat cultivars, that is, 'HUW 234' and 'HUW 468' and one known leaf rust susceptible cultivar , that is, 'Agra Local' were included as controls in the present study (Table 1). The purpose

was to characterize the effect of slow rusting genes on the disease severity. Three highly virulent leaf rust pathotypes [21R55 (104-2), 121R63-1 (77-5) and 29R45 (12-5)] were used in the study (Table 2). Their international designation is PHTTL, THTTS and FHTKL, respectively (Kolmer et al., 2007).

According to Bhardwaj et al. (2010), race 121R63-1 (77-5) is currently the most virulent and frequent race in the Indian subcontinent followed by 21R55 (104-2) and 29R45 (12-5).

Characterization of wheat germplasm lines for leaf rust resistance under field conditions

Pathological characterization of wheat genotypes for leaf rust resistance was done under field conditions. All the fifteen wheat genotypes were evaluated, each year under an induced epiphytotic condition in the field in three replications. Approximately, 55 to 60 plants of each genotype were grown in plots consisted of five rows of 3 m. The plant to plant and row to row distances were 20 and 25 cm, respectively. One row of 'Agra Local' was planted after 10 rows and in alleys to enhance the pressure of inoculums. Three plots of this design were prepared for three different rust pathotypes at the separate locations. Standard agronomic management practices were followed to raise a healthy crop.

The pathotypes 21R55 (104-2), 121R63-1 (77-5) and 29R45 (12-5) were used to inoculate the three plots separately located apart from each other at least 2000 m. All genotypes including spreader rows (Agra local) were inoculated at seedling stage in the field. The suspension of the three different pathotypes of *P. triticina* were separately prepared from the pure culture of the three isolates and uniformly applied on all genotypes by hand atomizer during the evening hours following the method of Chaurasia et al. (1999).

Disease assessment

The plants in all the three fields were observed regularly for measurement of latent period and incubation period. Slow rusting was characterized based on latent period, incubation period and effect of slow rusting genes on (%) disease severity and area under disease progress curve (AUDPC) values. After the appearance of disease symptoms, disease severity percent was measured

Table 2. All the three pathotypes used in the study and name of genes against which these pathotypes are effective.

S/no.	Pathotype	Effective against resistant gene(s)
1.	21R55 (104-2)	*Lr1, Lr3a, Lr13, Lr16, Lr17a, Lr23* and *Lr26* (Huerta-Espino et al., 2011)
2.	121R63-1 (77-5)	*Lr1, Lr2a, Lr2b, Lr2c, Lr3a, Lr10, Lr11, Lr14a, Lr14b, Lr15, Lr16, Lr17a, Lr20, Lr23, Lr26, Lr27+31, Lr33, Lr36, Lr38, Lr43* and *Lr44* (Bhardwaj et al., 2010)
3.	29R45 (12-5)	*Lr2b, Lr2c, Lr3b, Lr11, Lr12, Lr13, Lr14a, Lr14b, Lr15, Lr16, Lr18, Lr20, Lr21, Lr22a, Lr22b, Lr26, Lr30, Lr33, Lr35, Lr37* and *Lr38* (Datta et al., 2008)

according to Jeger (2004). Disease severity percent was recorded at three different stages *viz.*, late anthesis, late milking and dough stage. AUDPC based on disease severity over time, which has been suggested to be a pragmatic approach for disease assessment was estimated using the formula:

$$\text{AUDPC} = \sum_{i=1}^{i} [\{(Y_i + Y_{(i+1)})/2\} \times (t_{(i+1)} - t_i)]$$

Where, Y = disease level at time t_i ($t_{(i+1)} - t_i$) = time (in days) between two disease scoring.

Statistical analysis

Analysis of variance (ANOVA) for the disease severity percent at dough stage and AUDPC was performed using large spread excel sheet and SAS software (version 603; SAS Institute Inc; CaryNC 1997). The purpose of statistical analysis was to test the level of significance of disease reaction in comparison to highly susceptible genotype.

Differential reaction of wheat genotypes

Differential reaction was done with all the 15 genotypes by detached leaf technique in the controlled laboratory conditions following Nayar et al. (1997). Briefly, the leaf segments from all the 12 CIMMYT, two elite Indian wheat genotypes and 'Agra Local' were detached and put in water agar (WA) prepared in Petri plates. The leaf segments of the susceptible genotype 'Agra Local' were also placed in the Petri plates together following the methodology of Mikhailova and Kvitko (1970).

Three separate sets of experiments were prepared for each pathotypes. All the three sets of leaf segments were inoculated separately with the suspension of one of the three pathotypes. The Petri plates were sealed with the parafilm and incubated in Biochemical Oxygen Demand (BOD) incubator at 18°C temperature and 85% relative humidity and observation were recorded regularly for disease assessment.

Slow rusting was characterized based on latent period, incubation period and effect of slow rusting genes on (%) disease severity and AUDPC values. Latent period and incubation period reported to be a good parameter for evaluating disease resistance, were recorded against all the three pathotypes. Latent period is the state in which a host is infected with a pathogen but does not show any symptoms while, incubation period is the period of time between penetrations of a host by a pathogen to the first appearance of symptoms on the host. Disease severity was also observed and comparison was done between the most susceptible genotype, 'Agra Local' and each resistant wheat genotype included in the study.

Microsatellite analysis for the linked markers

Leaf samples were collected from 25 days old seedlings of 15 selected genotypes from field trial in poly bags. The leaf samples were frozen immediately in liquid nitrogen and stored in deep freeze (-80°C) for the purpose of genomic DNA isolation. Genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990). The concentration of DNA in each sample was determined and the working stock of concentration ~10 µg/µl was prepared for polymerase chain reaction (PCR).

The microsatellite markers were selected from the reference ITMI map (Röder et al. 1998; Ganal and Röder, 2007). A set of 10 linked SSR primers were custom synthesized by IDT, USA (Blaszczyk el al., 2004; Gultyaeva et al., 2009). The primers were dissolved in appropriate amount of Tris EDTA (TE) buffer according to the concentration of supplied primers, to make the working solution of 10µM concentration. Sequences and other information of the primers are given in Table 3. PCR reactions with ten SSR markers were performed as described by Röder et al. (1998) and Somers et al. (2004). DNA amplification was carried out in a 96 well thermocycler (Eppendorf Thermal Cycler, Germany) in a volume of 25 µl each containing 10 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, 10X PCR buffer and 1 U of Taq DNA Polymerase. The following PCR profile was followed: initial denaturation at 94°C for 3 min, followed by 45 cycles of 94°C for 1 min, 55 to 60°C (depending on primer pairs) for 1 min, 72°C for 2 min with a final extension step of 7 min at 72°C and the samples were held at 4°C until samples were taken out of the PCR for electrophoresis. The amplified PCR product was separated on 3% agarose gel at a constant voltage of 80 V for 2 to 3 h. The gel was stained with ethidium bromide solution, analyzed and photographed under visualized under gel documentation system

RESULTS

Field trials

The percentage disease severity at dough stage in all resistant genotypes was 0 to 20% in both the years, while it was 60 to 80% in the case of susceptible genotypes (Figure 1). AUDPC based on percentage disease severity data, recorded at three growth stages *viz.,* late anthesis, late milking and dough stages was between 50 to 350 in all the resistant genotypes, while it was between 1025.0 to 1362.5 in the case of susceptible genotype, that is, 'Agra Local' (Table 5). The data in relation to components of slow rusting resistance was statistically analyzed. The latent period and incubation period were significance differences between means of different parameters. The

Table 3. List of microsatellite associated with leaf rust used in present study.

S/N	Microsatellite marker	Linked with gene(s)	Distance from gene (cM)	Chromosome
1.	Xgwm295	Lr34	5.0	7D
2.	Xgwm1220	Lr34	0.1	7D
3.	Xswm10	Lr34	0.3	7D
4.	Xgwm272	Lr1	11.2	5D
5.	Xgwm654	Lr1	6.1	5D
6.	Xgwm33	Lr10	5.0	1A
7.	Xgwm382	Lr50	6.7	2A
8.	Xgwm18	Lr46	2.5	1B
9.	Xgwm495	Lr46	12.0	4B
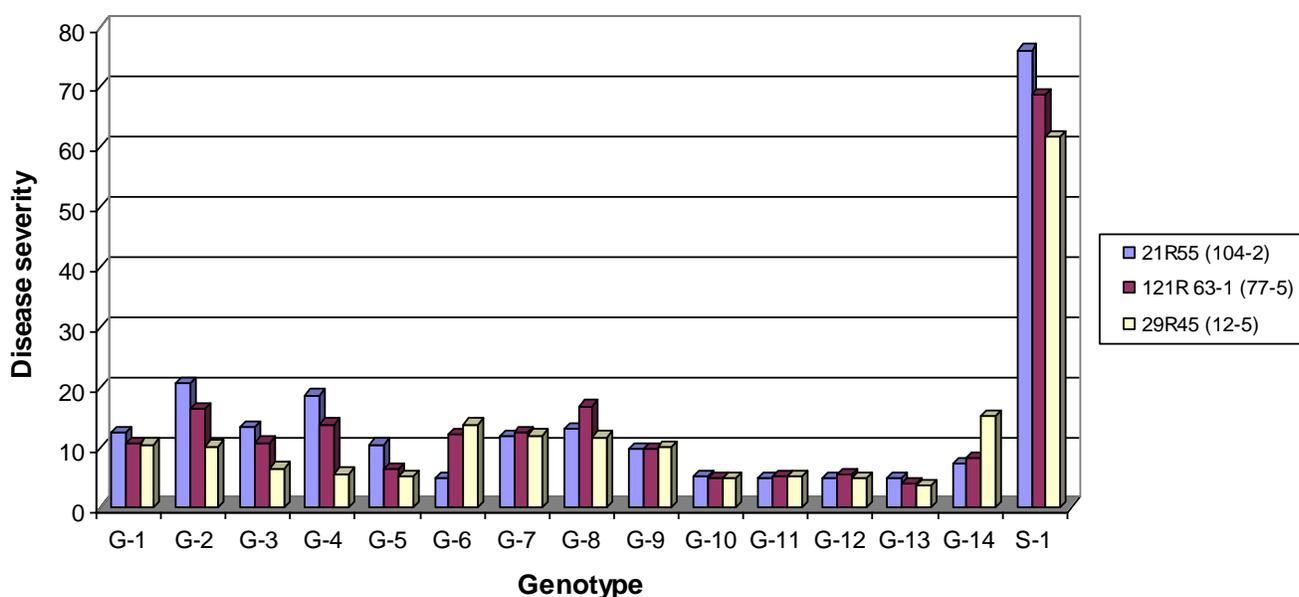
Figure 1. Mean disease severity per cent of all the fifteen genotypes against leaf rust pathotypes 21R55 (104-2), 121R63-1 (77-5), and 29R45 (12-5) are indicated by bars of different colours.

latent period and incubation period were significantly shorter on susceptible variety 'Agra Local' in comparing to the others (Table 4).

The mean latent period was longest on G-8 (13 days) followed by G-5 (12.67 days) and G-11 and G-14 (12.33 days in both the cases) against pathotype 21R55 (104-2). Against pathotype 121R63-1 (77-5), latent period was found to be highest on genotype G-12 (13.33 days) followed by G-5 and G-13 (13 days for both the cases) and G-6 (12.67 days). In case of pathotype 29R45 (12-5), the longest latent period was observed in genotype G-11 (14.33 days) followed by G-12 (14 days) and G-4 (13.67 days).

While, in case of pathotype 21R55 (104-2), genotype G-7 and G-12 showed 12.33 days of incubation period, which is longest among these 15 genotypes. This was followed by genotype G-11 and G-3, which had 12 and

11.67 days of incubation periods, respectively. The genotype G-13 had longest incubation period of 14 days against pathotype 121R63-1 (77-5). Genotypes G-5 and G-11 showed 13.67 days of incubation period. This was followed by genotype G-12, which had 13 days of incubation period. In the case of pathotype 29R45 (12-5), incubation period was found to be highest on genotype G-7 (13.67 days) followed by two genotypes G-4 and G-10 with 13.33 days of incubation. Genotype G-5 and G-11 had incubation period of 13 days.

Differential reaction of rust isolates on the wheat genotypes

All the 15 genotypes were also characterized for differential reaction using detached leaf technique (Nayar et al., 1997). Petri plates incubated in controlled laboratory conditions were regularly observed for latent

Table 4. Mean latent and incubation period exhibited by all the fifteen genotypes, when infected with pathotypes, 21R55 (104-2), 121R63-1 (77-5) and 29R45 (12-5).

Genotype	Pathotype 21R55 (104-2)				Pathotype 121R63-1 (77-5)				Pathotype 29R45 (12-5)			
	LP		IP		LP		IP		LP		IP	
	Mean	LSD	Mean	LSD	Mean	LSD	Mean	LSD	Mean	LSD	Mean	LSD
G-1	10.33±0.33	0.77	7.67±0.33	0.77	10.00±0.58	1.33	9.67±0.88	2.03	10.67±0.33	0.77	10.67±0.33	0.77
G-2	10.00±0.58	1.33	7.33±0.33	0.77	10.33±0.33	0.77	8.33±0.33	0.77	10.33±0.33	0.77	8.67±0.33	0.77
G-3	9.66±0.33	0.77	11.67±0.33	0.77	11.00±0.58	1.33	10.67±1.20	2.77	12.00±0.00	0.00	11.33±0.33	0.77
G-4	11.33±0.67	1.54	8.67±0.33	0.77	10.67±0.88	2.03	11.33±0.33	0.77	13.67±0.67	1.54	13.33±0.88	2.03
G-5	12.67±0.88	2.03	9.33±0.33	0.77	13.00±0.58	1.33	13.67±0.67	1.54	13.00±0.58	1.33	13.00±0.58	1.33
G-6	11.67±0.33	0.77	11.00±0.58	1.33	12.67±0.33	0.77	12.67±0.33	0.77	11.33±0.33	0.77	11.00±0.58	1.33
G-7	12.00±0.58	1.33	12.33±0.33	0.77	10.67±0.33	0.77	11.67±0.33	0.77	13.33±0.67	1.54	13.67±0.67	1.54
G-8	13.00±1.00	2.31	10.67±1.20	2.78	10.00±0.58	1.33	12.00±0.00	0.00	11.67±0.33	0.77	11.67±0.33	0.77
G-9	10.67±0.33	0.77	10.33±0.33	0.77	11.33±0.33	0.77	9.67±0.33	0.77	11.00±1.53	3.52	11.67±0.67	1.54
G-10	11.33±0.88	2.03	11.33±0.88	2.03	11.00±0.58	1.33	11.33±0.33	0.77	12.00±0.58	1.33	13.33±0.88	2.03
G-11	12.33±0.88	2.03	12.00±0.58	1.33	11.67±0.88	2.03	13.67±0.88	2.03	14.33±1.20	2.77	13.00±0.58	1.33
G-12	11.67±0.88	2.03	12.33±0.33	0.77	13.33±0.88	2.03	13.33±0.67	1.54	14.00±0.58	1.33	12.00±0.58	1.330
G-13	12.00±0.56	1.33	9.67±0.33	0.77	13.00±0.58	1.33	14.00±0.58	1.33	12.33±0.33	0.77	12.33±0.33	0.77
G-14	12.33±1.45	3.35	7.67±0.33	0.77	10.33±0.88	2.03	9.67±0.33	0.77	11.33±0.33	0.77	10.67±0.33	0.77
S-1	7.67 ±0.67	1.54	6.00±0.58	1.33	9.67±0.33	0.77	6.33±0.33	0.77	10.00±0.58	1.33	6.67±0.33	0.77

Table 5. Mean disease severity (%) and AUDPC for all the fifteen genotypes evaluated against three identified virulent pathotypes in the field during 2006-2007 and 2007-2008 *Rabi* seasons.

Genotype	Mean disease response to leaf rust											
	21R55 (104-2)				121R 63-1 (77-5)				29R45 (12-5)			
	% Disease severity		AUDPC		% Disease severity		AUDPC		% Disease severity		AUDPC	
	2006-2007	2007-2008	2006-2007	2007-2008	2006-2007	2007-2008	2006-2007	2007-2008	2006-2007	2007-2008	2006-2007	2007-2008
G-1	13.5±2.3	11.7±1.7	270.0±22.5	250.0±25.4	11.4±2.1	10.0±2.5	193.5±21.3	175.0 ±19.7	11.2±2.2	10.0±2.3	185.5±18.2	200.0±22.3
G-2	21.4±3.7	20.0±3.1	345.5±35.5	337.5±33.4	16.2±3.3	16.7±2.8	335.6±31.2	325.0 ±30.4	10.5±1.8	10.0±1.6	175.0 ±16.4	185.5 ±17.5
G-3	13.7±2.5	13.3±2.1	365.0±40.7	350.0±38.5	11.7±2.6	10.0±3.2	251.5±19.6	237.5 ±22.4	6.5±1.2	6.7±1.5	105.5±10.2	100.0±9.5
G-4	19.2±1.7	18.3±2.2	333.5±28.7	325.0±30.2	14.5±2.8	13.3±3.4	330.5±28.6	300.0 ±28.8	6.5±2.0	5.0±1.8	118.5±11.4	112.5±10.5
G-5	11.2±1.2	10.0±1.4	240.0±20.5	250.0±21.7	6.2±1.5	6.7±1.7	145.2±12.4	150.0±14.5	5.5±1.5	5.0±1.6	70.5±7.5	62.5±7.0
G-6	5.0±1.0	5.0±0.8	160.5±14.4	162.5±16.5	12.8±1.8	11.7±2.1	175.5±15.7	187.5±16.2	14.5±2.6	13.3±2.3	290.5±24.6	287.5±25.6
G-7	12.2±1.9	11.7±1.6	303.0±32.2	287.5±35.8	13.2±2.1	11.7±2.6	240.3±21.8	250.0±18.7	12.4±2.1	11.7±1.8	215.5±20.5	200.0±18.5
G-8	12.9±2.3	13.3±2.3	312.5±39.6	300.0±41.4	17.2±2.9	16.7±3.4	365.5±33.4	350.0±28.5	11.8±1.6	11.7±1.6	195.5±18.6	187.5±16.4
G-9	9.7±1.4	10.0±1.2	212.5±18.4	225.0±20.4	9.5±1.2	10.0±1.6	244.5±22.3	237.5±21.2	10.3±1.3	10.0±1.2	205.5±20.4	212.5±17.2
G-10	5.5±1.1	5.0±1.0	58.5±8.5	62.5±10.7	5.0±1.0	5.0±0.8	105.5±10.4	112.5±9.5	5.0±1.0	5.0±1.1	87.5±7.5	93.5±8.8

Table 5. Continued.

G-11	5.0±0.8	5.0±0.6	65.5±7.5	62.5±9.3	5.0±0.8	5.5±0.5	95.5±8.5	87.5±6.6	5.5±0.7	5.0±0.8	62.5±5.8	68.5±6.6
G-12	5.0±0.8	5.0±0.8	92.5±10.5	87.5±11.1	6.0±1.0	5.0±0.5	58.5±5.5	62.5±5.4	5.0±0.6	5.0±0.7	87.5±6.6	91.5±7.2
G-13	5.0±0.4	5.0±0.5	85.6±6.5	87.5±7.7	4.6±0.7	3.3±0.5	71.6±6.4	75.0±8.1	4.0±0.8	3.3±0.5	50.0±5.5	60.5±6.5
G-14	8.0±1.6	6.7±1.1	175.5±12.5	162.5±13.3	8.1±1.0	8.3±1.2	158.6±14.8	150.0±16.2	15.5±2.2	15.0±2.8	322.5±24.5	337.5±28.4
S-1	80.5±6.7	71.7±5.6	1550.5±180.6	1362.5±167.8	72.4±6.2	65.0±5.7	1205.3±96.2	1112.5±92.4	61.7±6.5	61.7±5.5	1105.0±98.8	1025.0±94.3

period and incubation period for all the pahtotypes. In case of pathotype 21R55 (104-2), genotypes G-4 and G-12 showed the longest latent period and the genotype G-11 showed the longest incubation period, while, genotypes G-10 and G-13 did not show any pustule growth. Genotype G-7 showed longest latent period against pathotype 121R63-1 (77-5) and in the same case, the longest incubation period was shown by genotype G-4, while other genotypes did not show any infection except control (Agra Local). Pathotype 29R45 (12-5) exhibited, the latent period was longest on G-6 and incubation period was found to be highest on genotype G-10, against the same pathotype where no infection was shown by other genotypes except control (Agra Local).

In addition, percentage disease severity for all the genotypes against three pathotypes was also recorded. The observations were recorded on the basis of total diseased leaf area. According to this observation, pathotype 21R55 (104-2) infected some genotypes *viz.*, G-2, G-6, G-7, G-8 and G-14 which showed few, small pustules, while genotypes G-10 and G-13 did not show any pustule on the detached leaves. However, infection was present on other resistant genotypes. While susceptible variety, that is, 'Agra Local' was completely burst and showed full susceptibility (Table 5). In case of pathotype

121R63-1 (77-5), the percentage disease severity was observed in genotypes G-2, G-4, G-6 and G-9. Some of the genotypes *viz.*, G-5, G-12, G-13, did not show pustules as well as any infection. In case of 'Agra Local', that is, the susceptible genotype, the leaves had full of pustules all over the leaf segment and had approximately 80% of disease severity. Pathotype 29R45 (12-5) somewhat showed less percentage disease severity. Genotypes, found to be more susceptible were G-1, G-2, and G-7 among the all15 genotypes except with pathotype 29R45 (12-5). Whereas, the genotypes which were close to near immunity were G-3 and G-5 which did not show even any infection on their leaves segments. In opposite to this, 'Agra Local' again observed with a very high degree of disease severity (Table 5).

On the basis of these tests, genotype G-10 and G-11 was considered most resistant genotypes, showed longest latent and incubation period. Some genotypes as G-5 and G-13, exhibited durable resistance against all the three pathotypes of leaf rust, that is, 21R55 (104-2), 121R63-1 (77-5) and 29R45 (12-5) and showed very high degree of resistance against leaf rust. Some other genotypes which expressed slow leaf rust resistance were G-3 and G-12. While susceptible variety (Agra Local) was complete burst and showed complete susceptibility against

all the three pathotypes. These results showed close proximity with the field evaluation conducted under natural conditions (Tables 4 and 5).

Molecular characterization of genotypes for slow leaf rusting genes, using SSR markers

Genotypes were further evaluated for the presence of various leaf rust resistant genes using linked microsatellite markers to confirm the introgression of slow rusting genes required to achieve near immunity. 10 SSR primers were used to analyze 15 wheat genotypes for the presence or absence of leaf rust resistant genes. All these primers produced distinct, reproducible bands/profiles either in all or some genotypes except control. This shows the positive linkage of leaf rust resistance with these SSR markers. Each reaction was repeated at least twice to control the reproducibility of the amplification pattern, without alteration in the protocol.

Out of 10 primers reported to be linked with leaf rust genes, eight produced gene specific fragments with all genotypes except the control, that is, 'Agra Local'. These primers were *Xgwm295*, *Xgwm1220*, *Xswm8* and *Xswm10* for *Lr34*, *Xgwm272* and *Xgwm654* for*Lr1*, *Xgwm33* for *Lr10*, *Xgwm18* and *Xgwm495* for Lr46 and *Xgwm382* for*Lr50*, Amplification in case of *Lr1*

Table 6. The fragment size (bp) of all fifteen wheat genotypes amplified with the ten SSR markers reported to linked with leaf rust genes.

S/N	Genotype	Primers and associated genes							
		Xgwm295	Xswm10	Xgwm272	Xgwm654	Xgwm33	Xgwm382	Xgwm18	Xgwm495
		(*Lr34*)	(*Lr34*)	(*Lr1*)	(*Lr1*)	(*Lr10*)	(*Lr50*)	(*Lr46*)	(*Lr46*)
1	G-1	244, 251	-	137	-	-	90,130	178,190	162
2	G-2	-	191	-	-	117	-	180,191	176
3	G-3	-	194	-	143	117	87,127	179,189	157
4	G-4	251	192	-	-	-	124	178,189	157
5	G-5	250	-	137	143	118,124	126	178,189	178
6	G-6	248	197	138	144	118,124	124	178,190	156
7	G-7	248	193	137	-	117	-	176,188	156,178
8	G-8	-	197	-	144	-	-	180,191	156
9	G-9	-	197	-	144	-	-	180,191	157
10	G-10	249	197	-	-	-	87,130	180,191	156
11	G-11	248	192,197	140	143	117	126	178,189	156
12	G-12	250	-	137	-	-	126	178,189	156
13	G-13	250	191	139	144	118	125	178,189	156
14	G-14	251	192	137	145	-	-	180,191	156
15	S-1	-		-	-	-	-	-	-

gene, two SSR markers *Xgwm272* and *Xgwm654* were reported to be linked with *Lr1* however, only *Xgwm654* showed the presence of *Lr1* in case of G-3, G-6, G-8 and G9 while, *Xgwm272* did not show the presence of *Lr1* in these genotypes. Similar pattern was observed in case of *Lr34* (four associated SSR markers are known) or *Lr46* (2 associated markers are known) where, only G-5, G-6, G-11 and G-12 showed the presence of all the associated markers which indicates that these are the different allelic forms of *Lr34* and *Lr46.* However, primer *Xgwm33* (linked with *Lr10*) did not amplify the expected fragment with genotypes; G-1, G-8, G-9, G-10, G-11, G-12 and G-13 whereas, no primer gave amplification with Agra Local, which showed the absence of leaf rust resistance genes in this susceptible variety. Thus, on the basis of this analysis we came to know that these primers were linked with leaf resistance and thus, considered as markers potentially related to the leaf rust resistant genes (Table 6). Allelic variation was observed in case of all the associated markers except *Xgwm1220*, which indicates the presence of different allelic forms of *Lr1, Lr10, Lr34, Lr46* and *Lr50* resistant genes among the resistant genotypes (Table 6). These allelic forms can be further pyramided for developing more resistant cultivars. Xgwm1220 which has been reported linked with Lr34, produced amplification in all the resistant as well as susceptible genotypes, that is, Agra Local.

On the basis of associated marker present, genotype G-5, G-11 and G-13 showed the presence of markers (10 markers) associated with all the variants of *Lr1, 10, 34, 46* and *50* genes followed by G-10 which showed the presence of seven markers linked with *Lr34, Lr46* and

Lr50 and G-12, showed the presence of six markers linked with *Lr1, Lr34, Lr46* and *Lr50* (Table 6).

DISCUSSION

Disease assessment revealed that genotypes G-5, G-10, G-11, G-12 and G-13 which showed slow rusting, had longer latent period and incubation period as well as reduced per cent disease severity. All these components can affect disease progress in the field. Latent period and incubation period have been found to be correlated with the
rate of rust development in the field (Johnson and Wilcoxson, 1979; Lehman and Shaner, 1992) and, therefore, are useful in characterizing the genetics of partial resistance in cereal crops. In some slow rusting component studies on leaf rust-bread wheat and leaf rust-barley (*Puccinia hordei*), latent period has been considered more reliable than uredinium size (Broers, 1989; Singh et al., 1991; Zadoks, 1971). Differences in slow rusting components found in different genotypes, that is, G-5, G-11 and G-12 against pathotypes 21R55 (104-2) 121R63-1 (77-5) and 29R45 (12-5), were possibly due to the influence of race specificity when host pathogen interaction is influenced by specific resistant genes (Southern and Wilcoxson, 1984), which is contrary to the observations of Kuhn et al. (1978), that slow rusting could be non race specific. The differential varietals response to existing virulent pathogens might be because of different gene combinations present in wheat genotypes. This mechanism is reported to be under the

control of several minor genes, which are present in different combinations in genotypes. Sometimes one additional gene can reduce the disease severity up to 50% (Singh et al., 1998). Investigation of partial disease resistance components are utilized across a range of plant pathogen systems (Onfroy et al., 2007; Sandoval-islas et al., 2007). The purpose of slow rusting or partial resistance or strategic deployment of race specific resistance is to achieve durable resistance, which remains effective in a cultivar during its widespread cultivation for a long sequence of generations or period of time in an environment favorable to a disease or pest (Johnson, 1988).

Detached leaf technique for differential reaction is considered as an efficient technique to characterize resistant genotype having different combinations of genes. Genotypes, G-4 and G-12 showed longest latent period in case of pathotype 21R55 (104-2), G-7 in case of pathotype 121R63-1 (77-5) and G-6 in case of pathotype 29R45 (12-5) and other genotypes did not show pustule growth *viz.*, G-5, G-10, G-11, G-12 and G-13, clearly indicate that these genotypes are effective against different pathotypes and the genotypes used in the study have different combinations of minor genes responsible for slow rusting components like latent period and incubation period.

Despite the fact that these three pathotypes are effective against 30 to 40 resistant genes, none of the resistant genotype showed out breakage of disease, which prove this theory that a combination of three to four or more than four resistant genes can provide durable resistance. Differential reaction results of G-5, G11, G12 and G13 exhibited less disease severity under controlled conditions also showed close proximity with field data which indicates that these genotypes have essential components of slow rusting (different allelic variants of slow rusting genes) required for durable resistance.
The objective of molecular characterization was to identify leaf rust resistance genes present in various combinations with the help of associated micro-satellite markers. On the basis of associated marker present, genotype G-5, G-11 and G-13 showed the presence of markers (10 markers) associated with all the variants of *Lr1, 10, 34, 46* and *50* genes followed by G-10 which showed the presence of seven markers linked with *Lr34, Lr46* and *Lr50*, and G-12, showed the presence of six markers linked with *Lr1, Lr34, Lr46* and *Lr50* (Table 6). Though, we used more than one linked SSR markers for various resistance genes but some of them did not produce polymorphic bands, for example, two SSR markers *Xgwm272* and *Xgwm654* were reported to be linked with *Lr1* however, only *Xgwm654* showed polymorphism and associated with the presence of *Lr1* in case of G-3, G-6, G-8 and G9 while, *Xgwm272* did give amplification in G-2, G-3, G-4, G8, G-9 and G-10 to show the presence of *Lr1* in these genotypes. Similar pattern was observed in case of *Lr34* (four associated SSR

markers are known) or *Lr46* (two associated markers are known) where, only G-5, G-6, G-11 and G-12 showed the presence of all the associated markers which indicates that these are the different allelic forms of *Lr34* and *Lr46*. *Xgwm1220* produced the marker band even in susceptible variety, that is, 'Agra Local' which indicates that this is not a true marker for *Lr34* and thus, not required to be included in marker-assisted selection (MAS). However, primer *Xgwm33* (linked with *Lr10*) did not give polymorphism with genotypes; G-1, G-8, G-9, G-10, G-11, G-12 and G-13 whereas, other primer either gave no amplification or the amplified fragment was not linked with *Lr* gene in Agra Local, which showed the absence of leaf rust resistance genes in this susceptible variety. On the basis of this analysis we came to know that these primers were linked with leaf resistance and thus, considered as markers potentially related to the leaf rust resistant genes (Table 6). Allelic variation was observed in case of all the associated markers except *Xgwm1220*, which indicates the presence of different allelic forms of *Lr1, Lr10, Lr34, Lr46* and *Lr50* resistant genes among the resistant genotypes (Table 6). These allelic forms can be further pyramided for developing more resistant cultivars. *Xgwm1220* which has been reportedly linked with *Lr34*, produced amplification in all the resistant as well as susceptible genotypes, that is, 'Agra Local'.

Near immunity was observed in G-5, G-11 and G-13 genotypes which may be due to various combinations of three to four adult and seedling plant resistance genes. Variation for disease resistance among resistant genotypes might be seen in the light of allelic variants of different resistant genes present in resistant genotypes. On the other hand, near immunity in case of genotypes G-5, G-11, G-12 and G-13 may be explained by the presence of potent resistant genes *Lr34* and *Lr46* in the background of 2-3 other minor resistant genes. After achieving a certain level of resistance, nature of gene is more important rather than number of effective genes. On the basis of field and laboratory evaluation, genotypes G-5, G-11, G-12 and G-13 had lower rate of disease development than other genotypes and the AUDPC was also lower than these genotypes which showed the presence of *Lr1, Lr10, Lr34, Lr46* and *Lr50* resistant genes. Therefore, it may be concluded that genes *Lr1, Lr10* and *Lr50* other than known slow rusting genes *Lr34* and *Lr46* also play a significant role to achieve near immunity and these genotypes may be regarded as slow rusting type. According to Singh et al. (2000), high yielding cultivars of bread wheat that were nearly immune to leaf rust and stripe rust could be developed by accumulating four or five slow rusting resistance genes through intercrossing parents that show intermediate disease levels.

The result further indicates that in addition to rate of disease development, it is imperative to consider the level of initial infection in selecting for partial resistance

using AUDPC. In addition, resistant genes characterized during the study are related to slow rusting and can be exploited for broadening the genetic base of cultivated wheat for rust resistance. The identification of closely linked molecular markers may lead to marker assisted mobilization of these genes in elite wheat backgrounds. The result also reveals that these resistant genotypes were developed with an objective to combine only minor/slow rusting genes so that out breakage of disease could be prevented even when new virulent races come in existence. These gene combinations are to provide near immunity/considerable level of disease resistance. Plant in such kind of resistance never show 100% resistance against prevailing race and thus, there is no selection pressure on pathogen population to evolve a new race to overcome the resistant gene(s). Since the genotypes *viz.,* G-5, G-11 and G-13 were showed near immunity against all the three virulent races effective against a number of resistant genes indicate that these genotypes have higher level of resistance may be included in breeding program in the development of more desirable cultivars.

Conclusions

Substantial variability was present among the resistant genotypes for leaf rust resistance. Since none of the genotype showed hundred percent resistance, therefore, resistance in present case was under the control of several minor genes, which can provide near immunity/higher level of resistance when come together in a genotype. Genotypes having all the five rust resistance genes, that is, *Lr1*, *Lr10*, *Lr34* and two slow rusting genes *Lr46* and *Lr50* and their allelic variants in various combinations, near immunity can be achieved. After achieving a certain level of disease resistance, the nature of particular gene and its contribution towards disease resistance is more important rather than number of additional genes. Combination of all these kinds of characterization *viz.,* morphological (per se performance), pathological (differential reaction) and molecular characterization with the help of associated micro-satellite markers is highly recommended to identify the near immune lines/genotypes. This was an initial study in this regards, more number of lines would be studied further following the same criteria.

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