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EVALUATION OF SEEDLING RESISTANCE AND MARKER ASSISTED SELECTION FOR LEAF RUST (*PUCCINIA TRITICINA*) RESISTANCE IN PAKISTANI WHEAT LANDRACES, CULTIVARS AND ADVANCED LINES

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ABSTRACT

Leaf rust is amongst major biotic constraints of wheat (*Triticum aestivum* L.) having ability to cause substantial yield reductions worldwide. A continuous exploration for novel sources of resistance is pre-requisite for its management. Objectives of study were to conduct resistance evaluation of 112 Pakistani landraces and 48 advanced lines/ cultivars at seedling stage with total 10 virulent pathotypes of leaf rust, 3 from Pakistan and 7 from U.S.A and to detect closely linked markers for *Lr10, 16, 34* and *67* genes through marker-assisted selection (MAS). Findings revealed most of Pakistani landraces showed lack of resistance at seedling stage. Only 7 accessions of landraces and 11 advanced lines were found highly resistant against all pathotypes of Pakistan. Similarly, 10 advanced lines exhibited high resistance while variability in resistance was recorded for landraces against all pathotypes tested from USA. Marker-assisted selection revealed *Lr* genes i.e. *Lr10, Lr16, Lr34* and *Lr67* were present at various frequencies. Highest frequency was observed for *Lr34* followed by *Lr16* & *Lr67* while lowest was recorded for *Lr10*. These genetic resources and lines identified effective against Pakistan and USA pathotypes are potential sources for improvement of leaf rust (LR) resistance and can be utilized as valuable material for breeding resistant wheat cultivars.

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INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is one of the most frequently cultivated, widely adapted (FAOSTAT and Nations, 2016) and second most after rice (Ortiz *et al.*, 2008). It represents around 19% of essential global cereal grain crop production (Todorovska *et al.*, 2009). The crop is currently prone to numerous challenges like biotic constraints which cause yield reductions (Gowda *et al.*, 2014). Three rust species are amongst the biotic constraints including brown rust caused by *Puccinia triticina*; black rust caused by *P. graminis* whereas *P. striiformis* is causative agent of yellow rust adversely affect quality of wheat grain (Chen, 2005; Bariana *et al.*, 2007; Ellis *et al.*, 2014; Singh *et al.*, 2015) and the final yield. These rust pathogens can escalate into major epidemics resulting extensive economic losses leading to crop failures, under favourable conditions. Occurrences of

colossal economic and yield reductions have been documented since ancient times (Wellings, 2011; Yamin *et al.*, 2021). Amongst these rust diseases, leaf rust has broad range occurrence in almost all wheat producing areas of the world (Huerta-Espino *et al.*, 2011). It is well adapted to broader climatic conditions and responsible for substantial yield and economic losses (Wamishe and Milus, 2004) in South America, Europe, Central Asia (Roelfs, 1992), in South Asia including Pakistan (Nagarajan and Joshi, 1985) and can be very serious in Great Plains of North America (Kolmer and Hughes, 2013). Due to broader climatic adaptation of different virulence phenotypes of this pathogen (Roelfs, 1992), long-lasting resistance has been difficult to achieve in the United States and across the globe. The environmentally friendly and most efficient method for decreasing damage caused by the pathogen, is cultivation of resistant cultivars (Oliver, 2014; Channa *et al.*, 2021).

Leaf rust resistance may be divided into two broad ranks of resistance namely adult-plant resistance (APR) and all-stage resistance (ASR) or seedling resistance (Chen, 2005). Seedling resistance is generally race-specific also known as monogenic resistance or vertical resistance present at all stages of plant growth, extends a high level of resistance (Chen, 2013) however it is frequently overcome by virulence variation of virulent pathotypes (Jin *et al.*, 2010; Kolmer *et al.*, 2013). Race-specific resistance is identified through varied range of hypersensitive reactions and contribute to involve levels of higher resistance as demonstrated by (McIntosh *et al.*, 1995). Conversely, adult plant resistance commonly known as horizontal resistance or partial resistance is more durable when deployed in combination (Singh *et al.*, 2011) . Race-nonspecific which is effective at later plant growth stages and can provide resistance to various prevailing strains (Lagudah, 2011). To date, more than one hundred resistance genes of wheat leaf rust have been reported while 72 of them permanently catalogued (McIntosh *et al.*, 2017). Of these, thirty-three genes have been shifted from other species into bread wheat (McIntosh *et al.*, 2013). Most of them confer hyper sensitive reactions, are race-specific resistance genes which have short-lived nature (Kolmer, 2013; Serfling *et al.*, 2011) often lose effectiveness and relatively few provide resistance (Lowe *et al.*, 2011) to the recent populations of pathogen. Wheat breeders are required to concentrate on the cultivars development having durable resistance against large genetic variation in pathogen populations causing frequent breakdown of leaf rust resistant varieties (Kolmer, 2005). Hence, it is dire need to explore new resistance sources to manage significant diseases of wheat.

To produce such cultivars equipped with new resistance sources, genetic resources with superior agronomic traits along disease resistance are required. Resistant genetic resources i.e. Landraces, synthetic hexaploid, elite, advanced lines, segregating population can be used to transfer superior agronomic traits and control rust diseases viz. leaf rust through breeding and biotechnological approaches. Among genetic resources wheat landraces have significant potential sources endowed with new resistance genes since comparatively few of them have been utilized in modern plant breeding (Reif *et al.*, 2005). Various studies have confirmed that wheat landraces can be effective resistance source of stripe, stem, and leaf rust (Zurn *et al.*, 2014). To validate combination of genes in potential donor and breeding material closely related molecular markers are great choice, modern swift and reliable approach. Wheat research has been revolutionized with development of next-generation sequencing and technologies of high-throughput genotyping (Trick *et al.*, 2012). Because of their cost-effectiveness (Mammadov *et al.*, 2012) high-throughput sequence-based markers like single-nucleotide polymorphism (SNP) (Wang *et al.*, 2014) and Diversity Arrays Technology have become suitable marker system and have provided a quick enhance in the detection of markers closely related with resistance to the disease attributes (Randhawa *et al.*, 2014). Hence, objectives of current work were to evaluate wheat landraces, advanced lines and cultivars for recognition of potential resistance at seedling stage against *P. triticina* and to identify leaf rust genes in Pakistani advanced lines/ cultivars, wheat landraces and cultivars through related molecular markers for breeding purpose.

MATERIALS AND METHODS

Wheat germplasm

In total, a collection of 160 genotypes comprising of 112 Pakistani wheat landraces and 48 Pakistani wheat cultivars/ advanced lines (Supplementary Table. 1) collected in Pakistan were received from USDA-Agriculture Research Service (ARS) National Small Grains Collections located at Aberdeen, ID, (USA) and United States Department of Agriculture (USDA)–

Agricultural Research Service (ARS), Cereal Disease Laboratory (CDL), Minnesota USA and Agronomy, Horticulture, and Plant Science Department South Dakota State University.

Experimental locations

These experiments were conducted under standard greenhouse conditions located at plant growth facility, Department of Plant Pathology, University of Minnesota, Saint Paul campus (USA).

Seedling test

For the seedling evaluation, three to four seeds of each plant material were grown in filled with plastic cones (5 x 18 cm; d x h) within 98 count racks (instead of peat pots) with 50:50 mix of steam sterilized field soil: Sunshine MVP potting mix (gypsum, Canadian sphagnum peat moss, nutrient charge, vermiculite, and dolomitic limestone) & (Sun Gro Horticulture, Quincy, Michigan). The cultivar Morocco considered as susceptible to all pathotypes of leaf rust was grown as a check along with a set of 24 near isogenic lines (NILs) deviated in single resistance gene of leaf rust. Infection types exhibited by near isogenic lines were utilized identity of the pathotypes and virulence and avirulence composition. Seedlings were planted rust-free environment at temperature cycle gradually change from 18^{o}C to 25^{o}C with 16 hour photoperiod.

Leaf rust pathotypes

A total of ten *Puccinia triticina* pathotypes, seven are currently prevalent pathotypes in the USA & three pathotypes were utilized in the seedling tests from Pakistan (Table 1).

Table 1. List of leaf rust isolates from U.S.A and Pakistan.

S. No	Pathotypes	Country	Year of Collection	Location	Host
1	RTPTPS	Pakistan	2016	Matli, Badin	Wheat
2	MNPSDS	Pakistan	2016	Matli, Badin	Wheat
3	MSPTDS	Pakistan	2016	Naudero, Larkana	Wheat
4	THBJ	USA	N/A	N/A	Wheat
5	TCRKG	USA	N/A	N/A	Wheat
6	KFDJ	USA	N/A	N/A	Wheat
7	TNBGJ	USA	N/A	N/A	Wheat
8	MCTNB	USA	N/A	N/A	Wheat
9	BBBD	USA	N/A	N/A	Wheat
10	PBMQQ	USA	N/A	N/A	Wheat

Inoculation

Eight seeds of the set of 160 genotypes consisting of 112 Pakistani wheat landraces and 48 Pakistani wheat cultivars and check rows of Morocco were grown individually in plastic cones within 98 count racks in greenhouse. To authenticate the spores viability (inoculated to the differential lines), check variety was utilized. The spores derived from individual pustule (12-15 mg of urediniospores/0.8 ml of Soltrol 170 oil, Phillips Petroleum, Bartlesville, OK using a custom inoculator pressured by a pump at 25-30 kPa) were dispersed in distilled water and utilizing atomizers sprayed onto (7-day-old) seedlings. Post inoculation, plants were humidified with fine droplets of distilled water produced with an atomizer and kept for twenty-four hours in dew chamber at temperature 18-22 ^{o}C and 90% relative humidity. Upon removing from dew chamber, to prevent possible contamination plants were positioned in isolated compartments in a greenhouse. Temperatures were kept between 18-25 ^{o}C in the greenhouse. Provision of natural light of the day was carried out for twelve hours per day with 120 µ E.M^{-2} S^{-1} photo synthetically active radiations released by cool white fluorescent tubes fixed over plants. Inoculation process was carried out according to the procedures followed by (Browder, 1971).

Incubation

Plants were incubated in a growth chamber (18-20 °C with 16 hour photoperiod supplied by 400 W high pressure sodium vapor lamps releasing 300 µmol photon s-1 m-2) after the infection period. No light was

supplemented to plants in the final phases of the infection period since pathogen can infect wheat without this treatment. After two additional hours, the chamber doors remained unlocked half-way to permit the surfaces of leaf to desiccate slowly before moving the plants back to the greenhouse conditions. Procedure of incubation was done as the method reported by (Parlevliet and Kuiper, 1977).

Disease assessment and scoring

Twelve to fourteen days after inoculation, the infection type (ITs) on plants were recorded using 0 to 4 scale developed by (DL and Kolmer, 1989). Accessions with infection types between 0 and 2 were documented resistant, while 3 and 4 scored recognized susceptible respectively (Table 2).

Table 2. Virulence/avirulence pattern of Pakistani and U.S wheat leaf rust pathotypes detected at seedlings of U.S near isogenic lines (NILs).

S. No	Pathotypes	Virulence	Avirulence
1	RTPTPS	1, 2a, 3a, 9, 16, 24, 26, 3ka, 17, 30, B, 10, 14a, 18, 21, 41, 14b, 20	2c, 11, 28, 42, 23
2	MNPSDS	1, 3a, 9, 24, 3ka, 17, 30, B, 10, 14a, 41, 3bg, 14b, 20	2a, 2c, 16, 26, 11, 18, 21, 28, 42, 23
3	MSPTDS	1, 3a, 9,16, 24, 3ka, 17, 30, B, 10, 14a, 18, 41, 3bg, 14b, 20	2a, 2c, 26, 11, 21, 28, 42, 23
4	THBJ	1, 2a, 2c, 3a, 30, B, 10, 14a	9, 16, 24, 3ka, 11, 17, 30,18
5	TCRKG	1, 2a, 2c, 3a, 26, 3ka, 11, 30, 10, 14a, 18, 28	9, 16, 24, 17, B, 21, 41, 42
6	KFDJ	2a, 2c, 3a, 24, 17, 30, 10, 14a	1, 9, 16, 26, 3ka, 11, B, 18
7	TNBGJ	1, 2a, 2c, 3a, 9, 24, 10, 28, 41	16, 26, 3ka, 11, 17, 30, B, 14a, 18, 21, 42
8	MCTNB	1, 3a, 26, 3ka, 11, 17, 30, B, 14a,	2a, 2c, 9, 16, 24, 10, 18, 21, 28, 41, 42
9	BBBD	20	1, 2a, 2c,3ka, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, B, 10, 14a, 18, 21, 28, 41, 42, 3bg, 14b, 23
10	PBMQQ	1, 2c, 3a, 3ka, 30, B, 10, 21, 28	2a, 9, 16, 24, 26, 11, 17, 14a, 18, 41, 42

Molecular markers

DNA Isolation

Entire set of genotypes was subjected to DNA extraction using a modified SDS Plant method as described (Edwards *et al.,* 1991). Assessment of DNA quantity and quality on 1% agarose gel & utilizing a Nanodrop spectrophotometer (Nanodrop Technologies). To utilize as functioning dilution for PCR amplification, DNA concentration regulated to a 10 ng/ul adding sterilized deionized and distilled water.

Genotyping of markers, PCR Assay and PCR amplification confirmation and denaturation

For molecular characterization of wheat genotypes and accessions, total 4 sequences flanking the linked SSR, SNP and STS microsatellite primer pairs were utilized (Table 3). SSR marker was amplified utilizing conditions reported by (Hiebert et al., 2010) and SNP genotyping (KASP- assays were developed for SNPs) was carried out as described by (Kassa *et al.,* 2017) while the method discussed by (Schachermayr *et al.,* 1997; Lagudah *et al.,* 2006) were applied to amplify STS markers. Gene Amp (R) PCR System 9700 Thermo-cycler was utilized for PCRs procedure. Performance of PCR reaction was done in 15µl mixture of reaction comprised of concluding concentration of 50mM KCl, 0.5 unit/µl of Taq DNA polymerase (Promega Madison WI, USA), 1X PCR Buffer, 0.4 uM each primer, 24ng/ul genomic DNA, 1.5 mM MgCl2 and 0.8 mM each of dATP, dGTP, dTTP and dCTP (Sigma Chemical Co., St. Louis, MO, USA).

Amplifications were designed, generally after 2 min of denaturation at 94 ℃ viz., two (for STS) and one (SSR) sequential cycles each comprising of 60 sec at 60 ℃, 60 sec at 50- 60 ℃ (contingent on the specific primers), 30 sec at 73oC & followed by step of extension at 73 ℃ of

05 min. Electrophoresis technique was applied for separating the products of PCR, in 0.4 mm × 50 cM, 4% polyacrylamide (1×TBE) buffer (0.002M EDTA, 0.089M Tris-borate and 0.089M boric acid) gels. A 211-bp fragment was successfully amplified for CFD23 PCR amplification. To evaluate the size of DNA fragment (each amplified) a ladder of 1KB bp step DNA was utilized. About 60 min, the gel was run at 100V stained with ethidium bromide (0.5µg/ml) & UV light was directed for photographed.

Table 3. Molecular markers used for the marker-assisted selection of leaf rust resistance genes.

Gene	Marker type	Name of Marker	Sequence of primer	References
Lr10	STS	Lrk10D1 & Lrk10D2	GAAGCCCTTCGTCTCATCTG TTGATTCATTGCAGATGAGATCACG	Schachermayr et al. 1997
Lr16	SNP	2BS-5175914_kwm847	TAG1-GTAACCACGGTGAAGCTGGCG TAG2-GTAACCACGGTGAAGCTGGCA TTGTTGTGCCGCCAGCCTCCAT	Kassa et al., 2017
Lr34	STS	csLV34	GTTGGTTAAGACTGGTGATGG TGCTTGCTATTGCTGAATAGT	Lagudah et al. 2006
Lr67	SSR	CFD23	TAG CAG TAG CAG CAG CAG GA GCA AGG AAG AGT GTT CAG CC	Hiebert et al., 2010

Electrophoresis & Visualization of gel

The PCR products were detected loading 10ul of the PCR product on 1.2% Agarose gels in 1X TBE buffer & UV light was applied for visualizing after ethidium bromide. Gel documentation (Digecel) system was used for presence and absence of gene (Doyle and Doyle, 1990).

Data analysis

Data on presence and absence of markers was collected after visualizing gel and comparing with band size of given markers linked to leaf rust resistance genes. The data was then subjected to frequency distribution and graphs were plotted accordingly.

RESULTS

Leaf rust resistance tests on Pakistani wheat landraces and cultivars

A set of 160 genotypes consisting of 112 Pakistani wheat landraces and 48 Pakistani wheat cultivars were utilized for evaluation of seedling resistance against leaf rust (*Puccinia triticina*). Total 10 wheat leaf rust (*Puccinia triticina*) races (7 races from U.S. and three Pakistani races) were used for seedling screening under controlled greenhouse conditions.

Frequency distribution of Pakistani wheat landraces response against US leaf rust pathotypes showed that fifteen (15) Pakistani wheat landraces were found highly susceptible against all (seven) tested US leaf rust pathotypes while sixty-two (62) landraces were susceptible against six pathotypes; twenty-eight landraces had susceptibility against five pathotypes; six landraces were found susceptible against four pathotypes and one landrace found highly susceptible and had high seedling infection types of 3+ against two pathotypes (Figure 1).

Frequency distribution response of Pakistani wheat cultivars and advanced lines against US leaf rust pathotypes showed that six (6) Pakistani wheat advanced lines/cultivars were found highly susceptible against all (six) US leaf rust pathotypes tested while twelve (12) advanced lines/cultivars found susceptible against five pathotypes; five (5) advanced lines/cultivars showed susceptibility against four; five (5) advanced lines/cultivars had high seedling infection types against three and eight (8) genotypes showed susceptibility against two while just two (2) advanced lines/ cultivars were susceptible against single pathotype whereas just ten (10) wheat genotypes had resistance against all tested US leaf rust pathotypes (Figure 1).

Frequency distribution response of Pakistani wheat landraces against Pakistani leaf rust pathotypes showed that ninety-four (94) of Pakistani wheat landraces are susceptible against all (three) pathotypes and eight landraces (8) were found susceptible against two pathotypes while three (3) landraces had (HITs of 3+) against one pathotype tested. Just seven (7) wheat landraces had resistance against all Pakistani leaf rust pathotypes tested (Figure 2). Frequency distribution response of Pakistani wheat genotypes against Pakistani

leaf rust pathotypes showed that twenty-one (21) Pakistani advanced lines/ wheat cultivars were found highly susceptible against all (three) Pakistani leaf rust pathotypes tested; eight (8) advanced lines/cultivars found susceptible against two pathotypes whereas eight (8) advanced lines/cultivars showed susceptibility against one pathotype tested and had seedling (HITs of 3+). Just eleven (11) wheat advanced lines/ wheat cultivars had resistance against all Pakistani leaf rust pathotypes tested (Figure 2).

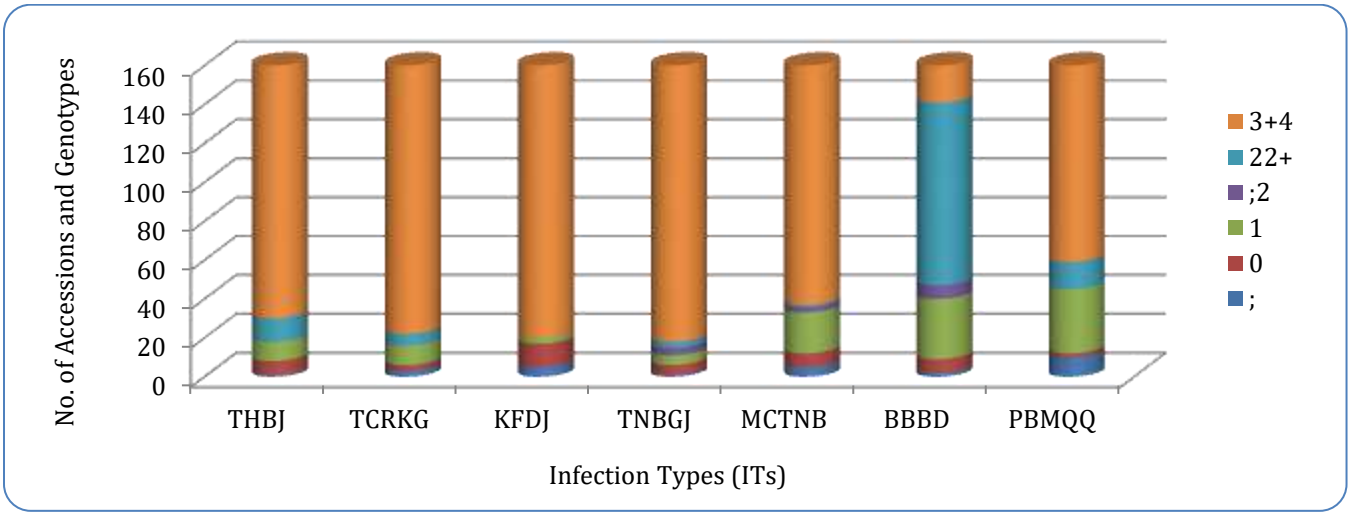

Figure 1. Frequency distribution of Pakistani wheat accessions and genotypes response against US leaf rust pathotypes.

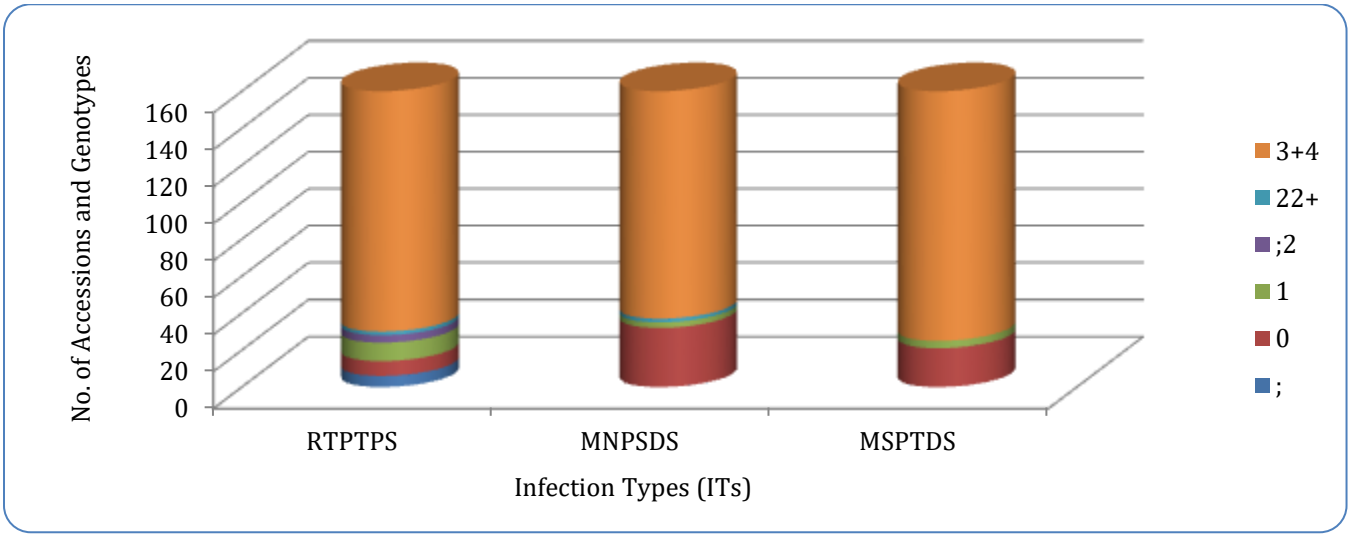

Figure 2. Frequency distribution of Pakistani wheat accessions and genotypes response against all Pakistani leaf rust pathotypes.

Molecular markers

Total four markers were utilized (STS, SNP and SSRs) to find out the absence/presence of *Lr10, Lr16, Lr34* and *Lr67* leaf rust resistance genes in a set of 160 genotypes comprising (112 Pakistani wheat landraces, 48 wheat cultivars and advanced lines). DNA markers primers Lrk10D1 & Lrk10D2, 2BS-5175914_kwm847, csLV34 and CFD23 gave reproducible results and succeeded to amplify fragments. These markers were used to detect leaf rust resistance genes *Lr10, Lr16, Lr34* and *Lr67* which produced 282bp, 826bp, 150bp and 211bp fragments known to be associated with and diagnostic for the presence of the leaf rust resistance genes *L10, Lr16,*

Lr34 and *Lr67* respectively.

STS marker Lrk10D1 & Lrk10D2 is dominant marker that was assayed for absence/ presence of *Lr10* gene. Frequency distribution showed that 12.5% percent wheat landraces/genotypes (20) resulted in amplification of 282bp fragment for marker Lrk10D1 & Lrk10D2 which is associated with the presence of *Lr10* gene (Figure 3).

Result revealed that 87.5% landraces/genotypes showed no desired band indicating lack of *Lr10* gene. Results of the marker Lrk10D1 & Lrk10D2 are presented in (Figure 3). Whereas SNP marker 2BS-5175914_ kwm847 was utilized for detecting the presence and absence of *Lr16* gene. Frequency distribution showed that current marker amplified successfully (826bp) desired fragment with percentage of 30.6% wheat landraces/ genotypes (49) that are associated with the presence of *Lr16* gene (Figure 4).

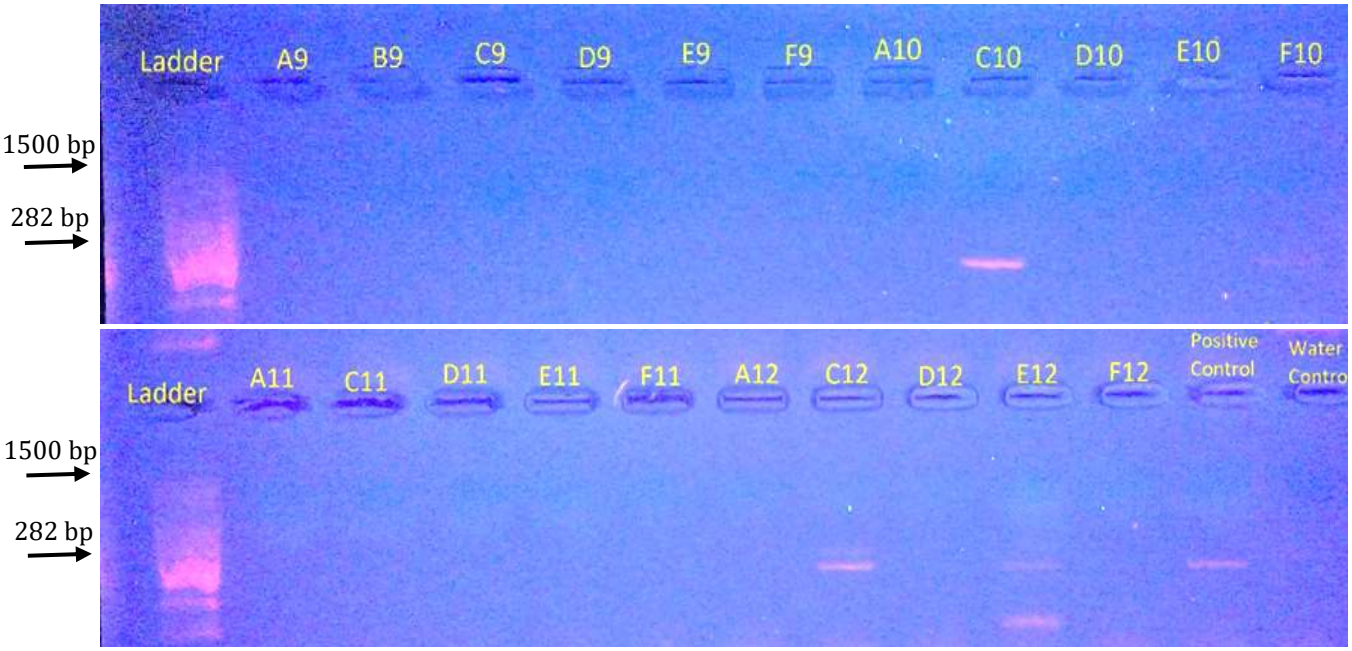

Figure 3. Polymerase chain reaction amplification products from wheat landraces, wheat cultivars and advanced lines using STS marker Lrk10D1 & Lrk10D2, arrow shows 282bp fragment indicating presence for *Lr10* gene. Ladder= Molecular weight Marker1Kbp 1, Positive control. C10=RCA-1, C12=V07096, E12=V-11160.

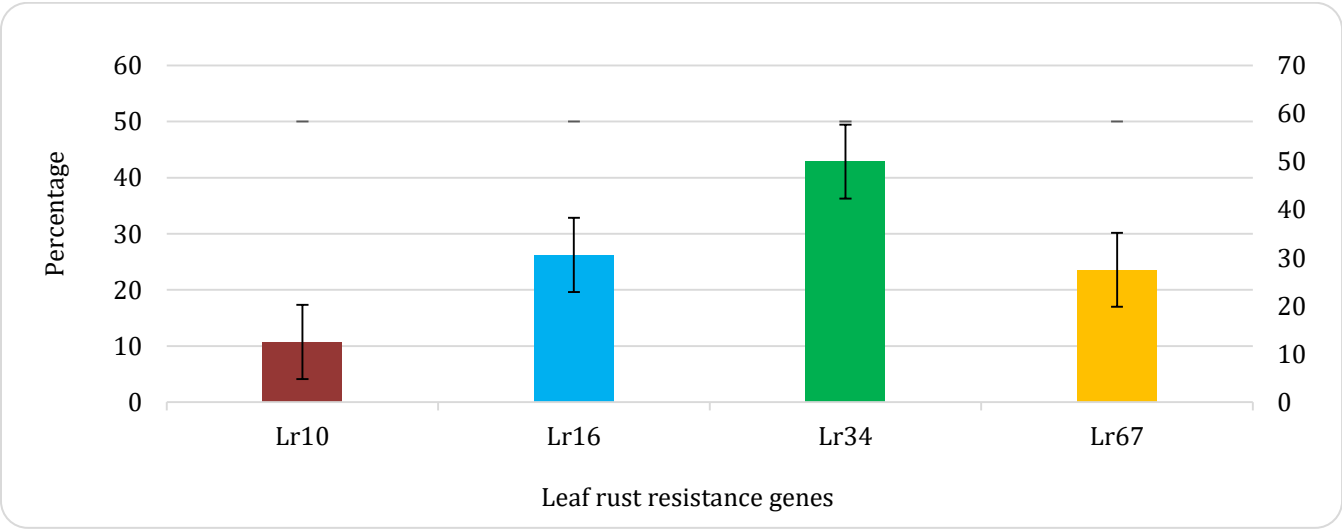

Figure 4. Frequency distribution of leaf rust resistance genes detected through marker assisted selection.

Result revealed that 69.4% of the landraces/genotypes showed lack of desired band indicating lack of *Lr16* gene. Results of the marker 2BS-5175914_kwm847 are presented in (Figure 5). While STS marker csLV34 was used for detecting presence and absence of *Lr34* gene. Frequency distribution showed that 50% percent wheat landraces/genotypes (80) resulted in amplification of 150bp fragment for csLV34 that is associated with the presence of *Lr34* gene. Result revealed that no desired fragment band was observed for rest of 50% landraces/genotypes indicating lack of *Lr34* gene. Results of the marker csLV34 are presented in (Figure 6).

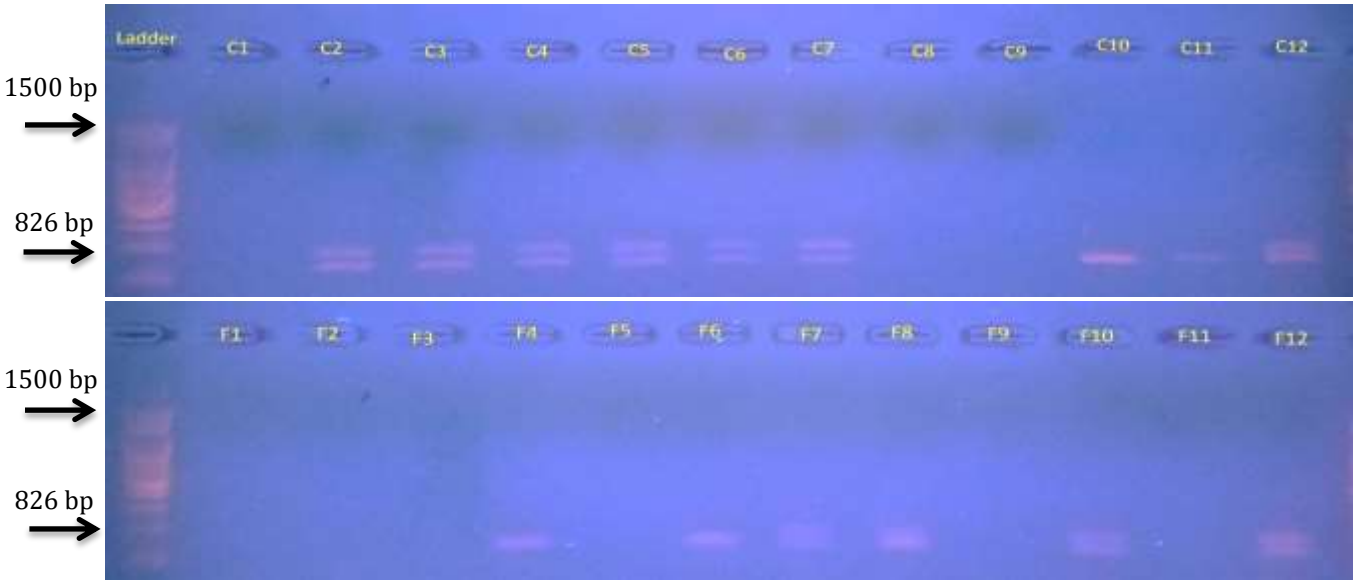

Figure 5. Polymerase chain reaction amplification products from wheat landraces, wheat cultivars and advanced lines using SNP marker 2BS-5175914_kwm847arrow shows 826bp fragment indicating presence of *Lr16* gene. Ladder= Marker1Kbp. C2=DN-93, C3=SKD-II, C4=SD-998, C5=99114, C6=TW06010, C7=Guard-C, C10=NR-421, C11=RCA-1, C12=99172, F6=Shalakot-13, F7=FSD-08, F8= Benzair-13, F10=Galaxy, F12=AAS-11.

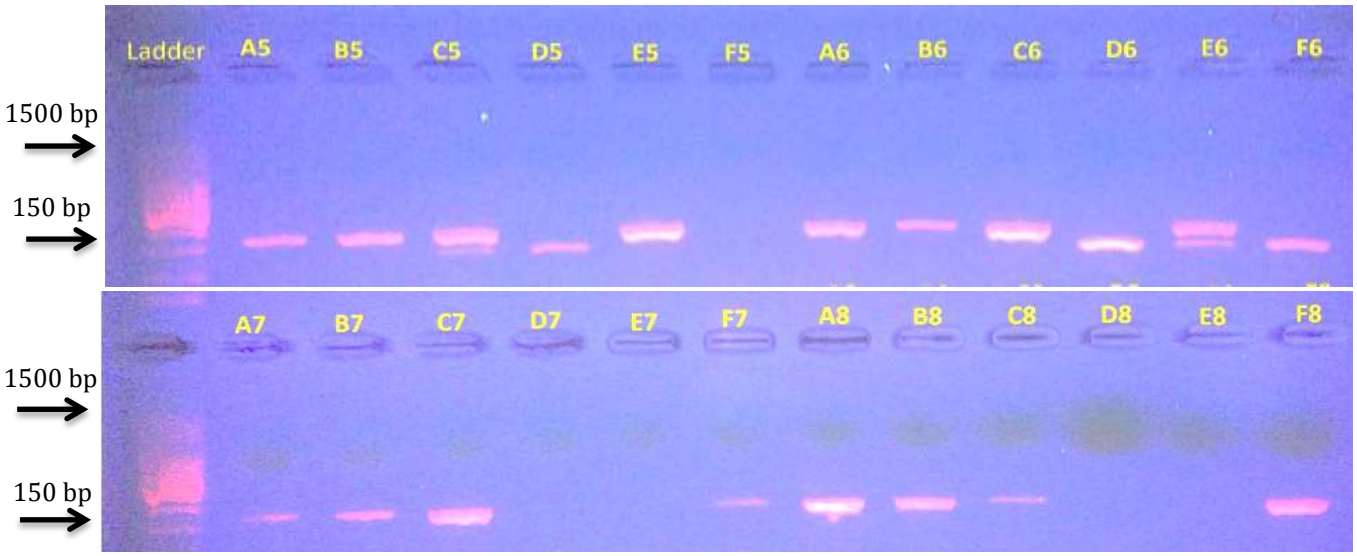

Figure 6. Polymerase chain reaction amplification products from wheat landraces, wheat cultivars and advanced lines using STS marker csLV34 arrow shows 150bp fragment indicating presence of *Lr34* gene. Ladder= Marker1Kbp. A5=PI 270016, B5=PI 388216, C5=99114, D5=V-10110, E5=NIA-MN-08, A6=PI 270022, B6=PI 520333, C6=TW96010, D6=V-10110, E6=Pirsabak-13, F6=Shalakot-13, A7= PI 270023, B7=PI572784, C7=Guard-C, F7=Faisalabad-08, A8=PI 270038, B8=PI 572840, C8=NR-421, F8=Benazir-13.

Whereas SSR marker CFD23 was analyzed for detecting the presence and absence of *Lr67* gene. Frequency distribution showed that CFD23 marker amplified successfully 211bp desired fragment with percentage of 27.5% wheat landraces/genotypes (44) that is associated with the presence of *Lr67* gene (Figure 7). Result revealed that 27.5% of the landraces/genotypes showed no desired band indicating lack of *Lr67* gene. Results of the marker CFD23 are presented in (Figure 7). Total fourteen (14) entries including three (3) wheat landraces viz. PI 270022, PI 270023, PI 572784 and eleven (11) wheat cultivars/advanced lines viz. 99114, TW96010, Guard-C, RCA-1, V07096, NIA-MN-08, Pirsabak-13, V-11160, Faisalabad-08, Benazir-13, Galaxy-13 resulted in amplifications of all four 282bp, 826kb, 150bp and 211bp fragments including two STS (Lrk10D1 & Lrk10D2 and csLV34), one SNP (2BS-5175914_kwm847) and one SSR (CFD23) markers which are associated with the presence of *Lr10*, *Lr16, Lr34* and *Lr67*.

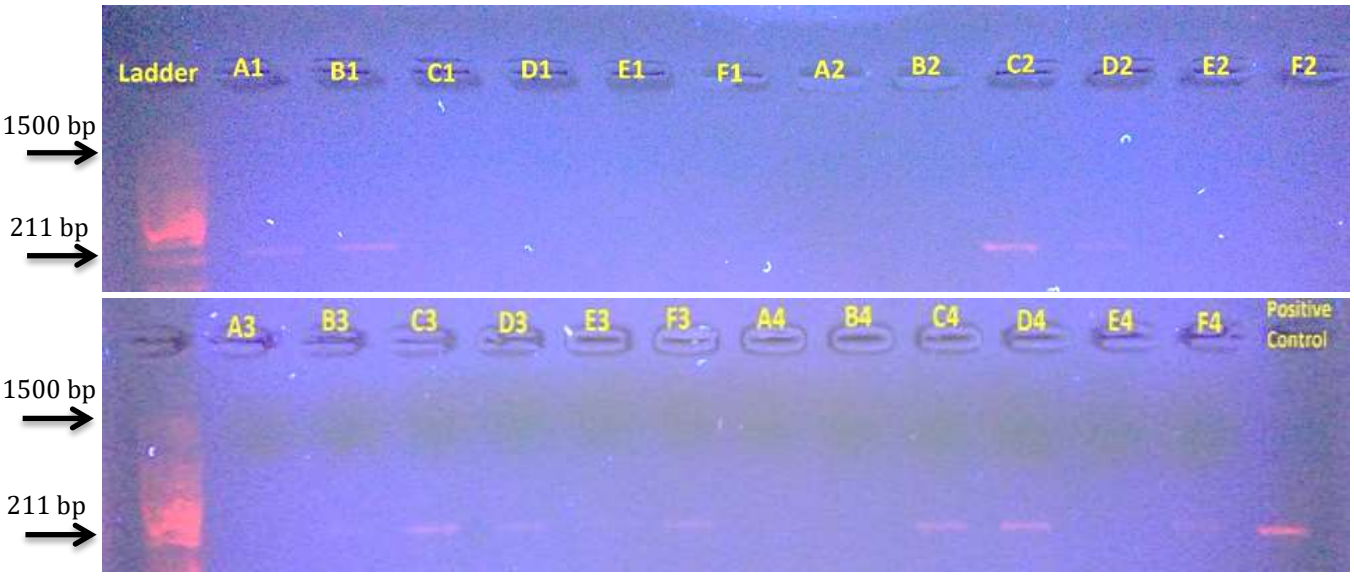

Figure 7. Polymerase chain reaction amplification products from wheat landraces, wheat cultivars and advanced lines using SSR marker CFD23, arrow shows 211bp fragment indicating presence for *Lr67* gene. M= Marker1kbp Ladder. A1=PI 269999, B1=PI 323342, C2= DN-93, C3=SKD-11, D3=V-10104, E3= NN-Gandum I, F3=Tijaban-10, C4=SD-998, D4=SAWSN-02-102, F4= Zardana.

Comparative study of marker and seedling results showed that among fourteen (14) entries amplified and described above except PI 270023 accession with (HITs) all other accessions and cultivars possibly containing all tested seedling resistance genes (*1, 2a, 2c, 3a, 9, 16, 24, 26, 3ka, 11, 17, 30, B, 10, 14a, 18, 21, 28, 41, 42, 3bg, 14b, 20* and *23*) as they had low infection types (ITs 0 to 2) to all tested pathotypes. Almost all of pathotypes used in this seedling test were found virulent *Lr3a, 3bg, 14b* and *20* while avirulent *Lr42, 23* genes. Among pathotypes BBBD and MCTNB were found avirulent to *Lr10* gene and presence of this gene and other three *Lr16, 34* and *67* in tested germplasm was validated by particular molecular markers identified to be linked to resistant genes. Indeed, leaf rust pathotypes that were utilized in current study were not sufficient to identify all of leaf rust (seedling) genes which were existed in accessions and cultivars or breeding lines. To identify additional genes, further studies may be conducted with more diverse collection of pathotypes and with help of marker assisted selection the presence of those leaf rust genes may be confirmed/validated.

DISCUSSION

Wheat landraces generally comprise collections from distinct geographical regions, which perform as a great source of new rust resistant genes for creating novel and genetically distinct disease resistant germplasm (Sthapit *et al.*, 2014). These were well recognized as valuable genetic resources offering resistance against leaf rust (Van Ginkel and Rajaram, 1993) and considered as an

essential genetic resource with well adaptation to numerous climatic conditions (Dotlačil *et al.*, 2010) and before green revolution were cultivated around the entire world. Development of genetically diverse cultivars with resistance to leaf rust disease is an important step to cope with new virulence phenotypes produced by rust pathogens frequently overcome ASR genes. Current study was designed to screen seedling resistance in Pakistani wheat landraces and cultivars and to detect leaf rust resistance genes in Pakistani wheat landraces through molecular markers. Closely linked molecular markers can assist the designing of gene combinations in potential donor sources and breeding material. Functional markers (FMs) are the highly advantageous markers for wheat breeding strategies and high-throughput genotyping for FMs could offer a colossal opportunity to efficiently practice marker-assisted selection while breeding cultivars.

Data indicated that pathotypes tested for exploring the resistance in the Pakistan wheat landraces and cultivars or advanced lines had a wide virulence spectrum. Hence, the seedling analysis of wheat landraces, cultivars and advanced lines exhibited lack of seedling resistance as the majority of the landraces and cultivars were recorded with susceptibility at the seedling stage. These genotypes displayed enormous potential for seedling resistance against the leaf rust pathogen under greenhouse conditions. However, result revealed that seven wheat landraces (PI 181087, PI 210900, PI 210903, PI 210904, PI 220072, PI 270042 and PI 572784) and eleven wheat cultivars and advanced lines (Shalakot-13, Faisalabad-08, Benazir-13, Sarsabz, Galaxy-13, Seher-06, Aas-11, TW96018, NR-409, Guard-C and NARC-11) were recognized with seedling resistance against all pathotypes tested from Pakistan. Result demonstrating these genotypes might possess a combination of seedling resistance genes. Likewise, ten wheat cultivars and advanced lines (Shalakot-13, Faisalabad-08, Benazir-13, Sarsabz, Galaxy-13, Seher-06, Aas-11, Guard-C, TW96018 and SRN 09111) were identified with seedling resistance while variability in resistance response was recorded for wheat landraces against all pathotypes tested from United States. Marker-assisted selection created very promising results in facilitating new gene deployment and gene pyramiding for swift release of rust-resistant varieties. Molecular markers like CAPS and SCAR or STS are existing for the resistance genes of leaf rust *Lr1, Lr9, Lr10, Lr16, Lr19, Lr21, Lr24, Lr25, Lr28, Lr29, Lr34, Lr35, Lr37, Lr39, Lr47, Lr50, Lr51* and *Lr67* ((Blaszczyk *et al.*, 2004; Obert *et al.*, 2005; Todorovska *et al.*, 2009; Herrera-Foessel *et al.*, 2011). Therefore more recently developed types of closely linked markers used to characterize or identify leaf rust resistance genes in tested accessions and genotypes.

Lr10 is a leaf rust resistance gene, derived from hexaploid wheat gene pool and is located on chromosome 1AS (Feuillet *et al.*, 1997; McIntosh *et al.*, 2003). It is found in most old Australian wheat cultivars, present in North American wheat cultivars and lines derived from the CIMMYT (International Maize and wheat Improvement Center) wheat breeding strategy. In addition, it is also postulated in Pakistani wheat cultivars (Mirza *et al.*, 2000; Rattu *et al.*, 2010) but high virulence to this gene is existing in Pakistan (Rizvi *et al.*, 1984; Hussain *et al.*, 1980). To detect this resistance gene in the wheat genome, functional markers were designed (Feuillet *et al.*, 2003). For the detecting absence/ presence of *Lr10,* we used the STS marker, Lrk10D1 & Lrk10D2. Successful amplification was recorded for the marker tested which detected the presence of gene in the landraces and cultivars/lines. Data comparison showed that twenty Pakistan wheat landraces/genotypes recorded with the presence of *Lr10* with 12.5 percent by STS marker primer Lrk10D1 & Lrk10D2. *Lr16* is a resistance gene of leaf rust, derived from *Triticum aestivum* (McIntosh *et al*., 2003) effective at the seedling stage (McCallum and Seto-Goh, 2003). It has been mapped to the terminal position of wheat chromosome arm 2BS (McCartney *et al.*, 2005) tightly linked with the resistance gene *Lr16* and had better resistance when deployed with gene *Lr34* in combination. Kompetitive Allele Specific PCR (KASP) assays were developed for all detected SNPs. The identifying potential of the SNPs co-segregating with *Lr16* was analyzed in a diverse set consisting of 160 landraces, cultivars and advanced breeding lines. SNP marker which showed consistency with the *Lr16* phenotype was exactly predictive of *Lr16* for forty-nine (49) cultivars/wheat breeding lines. Postulation of gene *Lr16* has been reported in wheat cultivars/ lines in Pakistan by several workers (Hussain *et al.*, 1998; Mirza *et al.*, 2000; Khan *et al.*, 2002). Marker assisted selection in landraces and cultivars showed that (30.6%) wheat landraces/genotypes contain *Lr16* gene.

Lr34 is a leaf rust resistance gene which is detected on

chromosome 7DS (Schnurbusch *et al.*, 2004a; Schnurbusch *et al.*, 2004b) was originally identified in spring wheat material at the CIMMYT (Singh and Rajaram, 1992; Singh, 1992) considered as key source of durable resistance (Roelfs, 1988). In addition, it is capable of acting synergistically with other (*Lr*) resistance genes (German and Kolmer, 1992) and pleiotropic effect on various diseases (Spielmeyer *et al.*, 2003). Presence of this gene has been reported by researchers in different countries viz., South American, Italian, Chinese (Dyck & Samborski, 1970) and Egyptian wheat (Imbaby et al., 2014). Current study also confirmed the presence of *Lr34* gene in 50% wheat landraces, cultivars and advance lines of Pakistan. Data analysis showed that STS marker primer csLV34 (for *Lr34*) produced highest (80) number of bands than any other marker and resulted in amplification of 150bp fragment. *Lr67* is a resistance gene of leaf rust, originated from *Triticum aestivum* (SI and WM, 2015), successfully mapped to chromosome 4D (Hiebert *et al.*, 2010) and is one of non-specific genes which are most frequently introduced genes in wheat globally (Haile and Rouml, 2013). To detect high levels of durable APR to brown rust and yellow rust in wheat, *Lr67*/Yr46 (slow-rusting genes) can be used in combination with other genes conferring slow rusting. Marker CFD23 was utilized for *Lr67* gene detection and the desired band (211bp) was successfully amplified with percentage of 27.5 wheat landraces/ genotypes (44), indicating presence of this gene in Pakistani wheat landraces/ genotypes. Evidences from results suggested that all closely linked markers tested exhibited strong association with *Lr10, 16, 34* and *67* and demonstrated their utilization in marker-assisted selection.

CONCLUSION

Major resistance genes have numerous disadvantages (Ayliffe *et al.*, 2008) and are still broadly utilized in wheat breeding for resistance. Marker-assisted selection can provide great facility for leaf rust gene transfer which is incumbent for resistance breeding. Marker-assisted selection revealed that leaf rust resistance genes *Lr10, Lr16, Lr34* and *Lr67* were present at various frequencies and successfully amplified with four closely linked markers. Total 9 accessions of wheat cultivars/advanced lines (Shalakot-13, Faisalabad-08, Benazir-13, Sarsabz, Galaxy-13, Seher-06, AAS-11, TW96018 and Guard-C) were found highly resistant against all pathotypes tested from Pakistan and USA. While 7 accessions of landraces (PI181087, PI210900, PI210903, PI210904, PI220072, PI270042 and PI572784) showed resistance against all pathotypes tested from Pakistan. This is suggested that genes (*Lr10, Lr16, Lr34* and *Lr67*) which have been detected in Pakistani landraces, advanced line/ wheat cultivars should be transferred through molecular breeding into modern varieties or susceptible bread wheat cultivars via conventional breeding approaches for the improvement of crop. There is necessity to broaden the genetic base of resistance by pyramiding multiple resistance genes of leaf rust. Further studies should be taken in near future on landraces for tracing other resistance genes that can be useful and deployed in Pakistani wheat cultivars for resistance against disease.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors contributed equally to this work.