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Awan, Shahid Iqbal; Ahmad, Syed Dilnawaz; Mur, Luis; Ahmed, Myhammad Shahzad

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**Full Length Article**

## Marker-Assisted Selection for Durable Rust Resistance in a Widely Adopted Wheat Cultivar “Inqilab-91”

Shahid Iqbal Awan<sup>1\*</sup>, Syed Dilnawaz Ahmad<sup>1</sup>, Luis A.J. Mur<sup>2</sup> and Muhammad Shahzad Ahmed<sup>3</sup>

<sup>1</sup>Department of Plant Breeding and Molecular Genetics, Faculty of Agriculture, The University of The Poonch, Rawalakot, Azad Kashmir-Pakistan

<sup>2</sup>Institute of Biological, Environmental and Rural Sciences, Edward Llwyd Building, Penglais, Aberystwyth University, Wales, United Kingdom

<sup>3</sup>Wheat Programme, Crop Sciences Institute, National Agriculture Research Center, Islamabad-Pakistan

\*For correspondence: shahidiqbal@upr.edu.pk

### Abstract

The slow rusting wheat cultivars viz., Tukur and Pavon-76 were hybridized with a widely adopted Pakistani wheat cultivar “Inqilab-91” to incorporate durable rust resistance. Specific primers were used to validate the presence of durable rust resistance in a bulked segregating population. The F<sub>1</sub> plants were backcrossed with either parent to obtain BC<sub>1</sub>F<sub>1</sub> populations which were then self-pollinated to get four F<sub>2</sub> populations. A total of 102 randomly selected back-crossed BC<sub>1</sub>F<sub>2</sub> plants were used for bulk segregant’s analysis. Genomic DNA was extracted from randomly selected individual plants and aliquots of DNA of each sample were mixed to constitute bulk pools of each population. Durable resistance gene *Lr34/Yr18* was heterozygous in populations 1, showing amplification of both the diagnostic *csLV34a* allele and the null *csLV34b* alleles. Population 2 amplified homozygous *csLV34a* alleles, while populations 3 and 4 amplified the null allele. The *Lr46/Yr29* gene was identified in population 3 and 4. Compared to Inqilab-91 the Population 1 indicated 33-67% and Population 2 indicated 56-78% lower infection type while population 3 and 4 indicated 22-44% lower infection type to yellow rust. All the populations and parents showed the prevalence of *Sr2* and absence of *Lr37/Yr17/Sr38* gene complex. © 2017 Friends Science Publishers

**Keywords:** Wheat; MAS; Rust; Durable Resistance; Inqilab-91

### Introduction

Hundreds of fungal disease resistance genes have been described in various cereal species. These genes can be generally classified based on their specificity and durability (Sucher *et al.*, 2016). Vertical resistance is quickly eroded as the rust virulent races being selected and increased in nature. The slow rusting genes that are weakly expressed at seedling stage but show optimum expression in the adult plant stage have also been identified. These genes express a moderate level of resistance, represented as smaller amount of uredinia of various sizes encircled by variable size of chlorotic tissue (Caldwell, 1968). The main feature of these genes in combination is that they present resistance to all identified rust races, with no established race specificity, while individually they don’t exhibit complete resistance. These genes also give long lasting durable rust resistance as their virulent forms have not yet been identified. Two of these genes, *Lr67* and *Lr34*, have been cloned and, respectively encode a hexose transporter (Moore *et al.*, 2015) and a putative ATP-binding cassette (ABC)

transporter (Krattinger *et al.*, 2009; Dakouri *et al.*, 2010; Sucher *et al.* 2016).

The well-known and categorized of all the race non-specific genes “*Lr34*” (Dyck, 1987) is present in wheat germplasm round the world (Kolmer *et al.*, 2008). Wheat varieties having *Lr34* also have a specific phenotype of leaf tip necrosis, expressed independent of rust infection. Though races virulent to *Lr34* have not been identified but the varieties containing *Lr34* alone don’t show elevated level of resistance. Supplementary genes for resistance are essential for wheat cultivars to express an effective resistance. Various genes in combination with *Lr34* have shown durable resistance. Hard red spring wheat varieties having combinations of *Lr13*, *Lr16*, *Lr23* and *Lr34* have shown modest to higher level of resistance for more than thirty years (Kolmer and Jin, 2007). Because of the durability and non-specificity of its resistance against multiple pathogens, *Lr34* has become one of the most important disease-resistance genes in wheat worldwide and has been utilized since the early 20th century (Fang *et al.*, 2017).

Some additional adult plant leaf rust resistance genes include *Lr68*, *Lr67* and *Lr46* (Singh *et al.*, 1998; Hiebert *et al.*, 2010). The gene *Lr46* is analogous to that of *Lr34* in its effect at adult plant stage, however at seedling stage it is weaker in effect compared to *Lr34* (Martinez *et al.*, 2001). Wheat cultivars having a combination of adult plant genes *Lr34*, *Lr46* and *Lr68* demonstrated durable type of resistance (Kolmer, 2013). Similarly, stem rust resistance in wheat provided by the gene *Sr2* have stayed effective around the world for over fifty years. The resistance conferred by *Sr2* is linked to highly varied disease symptoms; it is a recessive gene and expresses itself mainly at the adult plant stage (Spielmeyer *et al.*, 2003).

Developing a variety with durable resistance can be challenging as resistance is often quantitative in nature, and highly affected by the environment (Klarquist *et al.*, 2016). Molecular markers for the identification of these genes make the selection and handling of genetic stock easier during the course of a breeding programme. These markers are in fact the indicators of presence or absence of specific genes (William *et al.*, 1998). Among various molecular marker techniques microsatellites or simple sequence repeat (SSR) have been the markers of choice for identification analysis (Manifesto *et al.*, 1998; Szabo and Kolmer, 2007; Kolmer, 2013). These markers can be utilized to expedite the introgression of resistance genes into novel cultivars. When initiating a marker assisted selection (MAS) project for a trait of interest there are several considerations (Anderson *et al.*, 1993). Parental selection may also be complicated for MAS because different recurrent parents differ in their strengths and weaknesses in having various traits. The reasons to transfer slow rusting resistance genes into a popular variety in a planned fashion, instead of identifying a totally new variety are: (1) popular varieties have unique combinations of numerous genes to achieve their wide adaptability and acceptability by millions of farmers; (2) it is comparatively easy to add a few more genes in such cultivars; (3) a planned transfer ensures selection for slow-rusting genes-based resistance; and (4) the strategy is highly economical, as only a few crosses need to be made. In wheat marker assisted selection will be most efficient in  $F_2$  or first back cross because these are the generations of maximum segregation (Sorrells, 1998; Singh *et al.*, 2012).

The main objectives included in this study were (a) to transfer the adult plant resistance found in Tukur and Pavon-76 into a widely adopted wheat cultivar Inqilab-91, (b) to identify suitable segregating populations having slow rusting genes, (c) to screen the segregating populations for certain race specific genes using DNA markers to envisage the durability of rust resistance in the populations.

## Materials and Methods

Three wheat varieties Tukur and Pavon-76 and Inqilab-91 were used as parent material in this study. The data from

various rust trap nurseries specified that Tukur and Pavon-76 carrying slow rusting genes, *Lr34/Yr18* and *Lr46/Yr29* were effective when combined with other genes of rust resistance (Rattu *et al.*, 2012). Inqilab-91 was the most widely adopted and high yielding wheat cultivar previously grown in Pakistan but is now susceptible to rust having a race specific gene *Yr27*. The virulence of *Yr27* virulence is wide spread in this region (Hovmøller, 2013). Both *Lr34/Yr18* and *Lr46/Yr29* gene combinations from Tukur and Pavon-76 were exploited to hybridize with Inqilab-91.

## Hybridization Strategy

The slow rusting wheat varieties Tukur and Pavon-76 were crossed with susceptible Inqilab-91. The  $F_1$  plants were backcrossed with either parent to produce 4  $BC_1F_1$  populations. The plants of the  $BC_1F_1$  populations were selfed to produce  $BC_1F_2$  populations in Tukur and Pavon-76 back grounds as, Population 1: Tukur/Inqilab-91/Inqilab-91, Population 2: Tukur/Inqilab-91/Tukur, Population 3: Pavon-76/Inqilab-91/Inqilab-91 and Population 4: Pavon-76/Inqilab-91/Pavon-76. A total of 102 randomly selected back crossed  $F_2$  plants were used for bulk segregants analysis, including 24 plants from population 1, 45 plants from population 2, 17 plants from population 3 and 16 plant from population 4.

## DNA Extraction and Quantification

Leaf samples of each back crossed  $F_2$  population and their parents were collected from the field at Faculty of Agriculture, Rawalakot, Azad Kashmir and stored at  $-80^\circ\text{C}$ . The samples were taken to the Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales, United Kingdom for the extraction of DNA. DNA from each sample was extracted separately using standard protocol in a 2% hexadecyl trimethyl ammonium bromide (CTAB) buffer (100 mM Tris-HCl, 1.4 mM NaCl, 20 mM EDTA, pH 8.0). The DNA pellet was re-suspended in 50  $\mu\text{L}$  TE buffer (1 mM Tris, 1 mM EDTA, pH 8.0).

Genomic DNA of the samples and parents was quantified with the help of microplate spectrophotometer (Epoch, Program: Gen. 51.10). Ten times (10x) dilution of genomic DNA was made to increase their volume for the convenient mixing. Aliquots of genomic DNA containing 50 ng/ $\mu\text{L}$  DNA of each sample was mixed to constitute bulk pools of each population for bulk segregants analysis. The pools were quantified once again and 10x dilutions were made.

Purity of genomic DNA in each sample was counter checked by performing PCR with barcoding primers. The DNA samples showing missing/fuzzy bands were discarded and DNA extraction process was repeated.

## PCR Amplification and Analysis

Master Mix for each PCR reaction constituted 5.83  $\mu\text{L}$

biomix (Bioline), 5.72  $\mu\text{L}$  HPLC water, 0.041  $\mu\text{L}$  forward and reverse primer each. Total volume for each PCR reaction was 10  $\mu\text{L}$  comprising 9.5  $\mu\text{L}$  of master mix and 0.5  $\mu\text{L}$  of 50 ng/ $\mu\text{L}$  template DNA. The PCR reactions were performed in thermal cycler (My Cycler, BIORAD) following standard procedure. Specific conditions for each primer were optimized before carrying out the real experiment.

The primer sequences for the various DNA markers were assessed from the MAS website: (<http://maswheat.ucdavis.edu/Index.htm>). Genomic DNA was amplified with primers to validate the presence or absence of rust resistant genes in different populations and their parents.

PCR products of CAPS markers *csSr2*, *KSUD14 STS*, *S30-13* and *AGA7-759* were washed and re-precipitated before enzyme digestion. The PCR products of *csSr2* were cleaved with *BspHI* (for *Sr2*), *KSUD14 STS* with *MspI* (for *Lr21*) while that of *S30-13* and *AGA7-759* (for *Lr51*) were restricted with *BamHI*. Two  $\mu\text{L}$  of 10x reaction buffer and 0.5  $\mu\text{L}$  of *BspHI/MspI/BamHI* (Thermo Scientific®) were added to each re-suspended PCR product. The samples were incubated at 37°C for thirty minutes.

### Gel Electrophoresis

Electrophoresis was done to resolve the amplified products using BIORAD Wide mini-Sub Cell GT., apparatus. A 3% agarose gel was made by dissolving 2 g of high gelling agarose and 1 g of low gelling agarose powder in 100 mL of 1x TAE buffer. The gels were stained with 1  $\mu\text{L}$  of gel stain (Lonza GelStar™, Rockland, USA), 1  $\mu\text{L}$  of 6x loading dye (Promega) was added to 10  $\mu\text{L}$  PCR products and mixed by vortex. The wells were loaded with 10  $\mu\text{L}$  of samples. A 5  $\mu\text{L}$  of 100bp DNA ladder (Promega) was run along with the samples. The gels with variable bands were visualized using gel documentation system (BioRad, ChemiDoc™).

PCR products having alleles with smaller band differences were separated and visualized on 6% acrylamide gels using a vertical electrophoresis apparatus (BIORAD, Mini). The gels were run at 80V until blue line of the dye reached the bottom of the gel. Gel stain (GelStar™) was used for the detection of DNA on vertical gels by post staining. In 50 mL of 1x TAE buffer 5  $\mu\text{L}$  of gel stain was added. The stain was gently poured above the exterior of the gel, incubated for 30 min at room temperature and visualized on gel documentation system.

### Phenotyping of Parents and BC<sub>1</sub>F<sub>2</sub> Populations

All the BC<sub>1</sub>F<sub>2</sub> populations along with the parents were planted inside a trap nursery and scored for yellow rust (*Yr*) response. The cultivar Morocco was used as susceptible check. The yellow rust severity was recorded based on the modified Cobb scale (Peterson *et al.*, 1948).

### Results

The populations 1 and 2 derived after a single backcross from the parent Tukurú showed the presence of *Lr34/Yr18* allele. The varieties Inqilab-91, Pavon-76 and Populations 3, 4 derived from them showed *csLV34b* a null allele for *Lr34/Yr18*. Only Pavon-76 and the populations derived after a backcross from it (3 and 4) indicated the presence of *Lr46/Yr29* genes (Table 1). All the populations and parents showed Hope type allele of *Sr2*. The parents and the populations illustrated the absence of horizontal resistance gene *Lr37/Yr17/Sr38* (Fig. 2).

Under field conditions the plants in population 1 showed an infection type of 30-60 MRMS while those in population 2 showed 20-40 MRMS. In population 3 and 4 having *Lr46/Yr29* gene an increase of 22-44% resistance against yellow rust was observed (Fig. 3).

Presence/absence matrix for vertical resistance genes indicated that the genes *Lr32*, *Lr39* and *Lr51* were present in all the genotypes except Pavon-76. The stem rust resistance gene *SrCad* was present in Pavon-76. The gene *Lr50* was not detected in population 2 (Table 2). Tree diagram based on horizontal and vertical resistance genes indicated that Population 3 and 4 while Population 1 and the parent Tukurú were similar (Fig. 5).

### Discussion

#### Authentication of *Lr34/Yr18* in Inqilab-91 Derived Populations

Both the Tukurú derived populations (1 and 2) showed the presence of *Lr34/Yr18* allele (Fig. 2). The genes were in heterozygous form in population 1 showing the presence of both the alleles, i.e., 150bp *csLV34a* and 229bp *csLV34b* PCR products. The 229bp product related to *csLV34b* is a null allele for *Lr34/Yr18* (Kolmer *et al.*, 2008). The population 1 derived its *csLV34a* allele from the female parent Tukurú and *csLV34b* allele from male parent Inqilab-91. Careful selection is required utilizing morphological marker of leaf tip necrosis (LTN) to fix the *Lr34/Yr18* genes in this population. The Population 2 exhibited a smaller PCR product corresponding to a Tukurú derived *csLV34a* allele in homozygous form indicating that the durable resistance genes *Lr34/Yr18* were fixed in this population. Since the genes were fixed in population 2, the population 1 having the heterozygous allele may be discarded because it is segregating and stability concerns in succeeding generations.

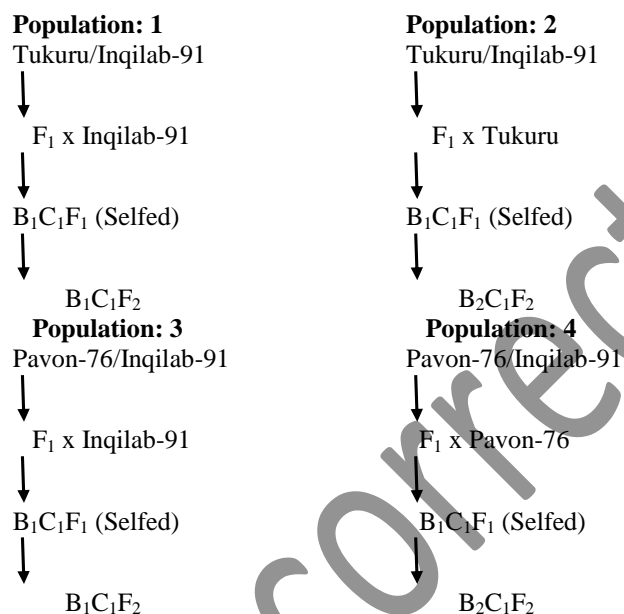
The populations 3, 4 and their parent cultivars Inqilab-91 and Pavon-76 showed *csLV34b* a null allele for *Lr34/Yr18*. Singh (1993) also reported the absence of *Lr34/Yr18* genes in Pavon-76. The varieties Jepatico-73R and Opatá-85 were positive checks for the presence of *Lr34/Yr18* genes produced *csLV34a*.

**Table 1:** Presence/absence matrix for durable rust resistance genes in parents and BC<sub>1</sub>F<sub>2</sub> populations of wheat

	<i>Lr34/Yr18</i>	<i>Lr46/Yr29</i>	<i>Sr2</i>	<i>Lr37/Yr17/Sr38</i>
Tukuru	+	-	+	-
Population 1	+/-	-	+	-
Population 2	+	-	+	-
Inqilab-91	-	-	+	-
Population 3	-	+	+	-
Population 4	-	+	+	-
Pavon-76	-	+	+	-

**Table 2:** Presence/absence matrix for race-specific genes in parents and BC<sub>1</sub>F<sub>2</sub> populations of wheat

	<i>Lr21</i>	<i>Lr32</i>	<i>Lr39</i>	<i>Lr50</i>	<i>Lr51</i>	<i>SrCad</i>	<i>Sr28</i>	<i>SrWeb</i>	<i>YrR61</i>
Tukuru	-	+	+	+	+	-	-	-	-
Population 1	-	+	+	+	+	-	-	-	-
Population 2	-	+	+	-	+	-	-	-	-
Inqilab-91	-	+	+	+	+	-	-	-	-
Population 3	-	+	+	+	+	-	-	-	-
Population 4	-	+	+	+	+	-	-	-	-
Pavon-76	-	-	-	+	-	+	-	-	-

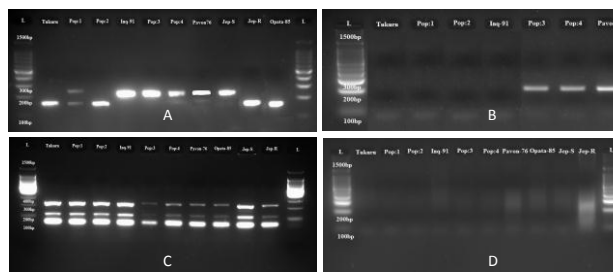


**Fig. 1:** Crossing plan to incorporate durable rust resistance in wheat cultivar Inqilab-91

Presence of *Lr34/Yr18* in the cultivar Opata-85 was also reported in the literature (Singh and Rajaram, 1992; Singh, 1993; Kolmer *et al.*, 2008). The negative check Jupetico-73S showed the presence of null allele for *Lr34/Yr18* (Table 1).

**Authentication of *Lr46/Yr29* in Inqilab-91 Derived Populations**

Only Pavon-76 and the populations derived from it (3 and 4) indicated the presence of *Lr46/Yr29* genes in homozygous form (Fig. 2). Both these populations preserved these genes



**Fig. 2:** PCR amplification for durable rust resistance genes in parents and BC<sub>1</sub>F<sub>2</sub> populations of wheat (A. *Lr34/Yr18*, B. *Lr46/Yr29*, C. *Sr2*, D. *Lr37/Yr17/Sr38*)

from Pavon-76. The genes were reported to be present in Pavon-76 by Singh *et al.* (1998) that had stayed resistant ever since its release in 1976. The cultivar Inqilab-91 which is the other parent of these populations showed the absence of *Lr46/Yr29* genes. The cultivar Tukuru and its derived populations (1 and 2) indicated the lack of *Lr46/Yr29* allele.

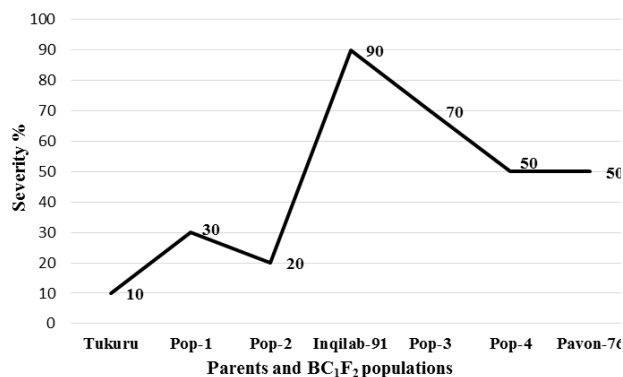
**Exploration of Additional Slow Rusting Genes**

**Validation of *Sr2*:** The marker *csSr2* obtained from the *Sr2* locus identifies 3 alleles of *Sr2* accurately. 1) 'null' allele or lack of amplification 2) Marquis type allele: two fragments (225bp and 112bp) following amplification and cutting by means of *Bsp*HI enzyme. This allele is linked to non-*Sr2* wheats 3) Hope type allele: three fragments (172bp, 112bp and 53bp) following amplification and cutting by means of *Bsp*HI enzyme. This allele is diagnostic to *Sr2* (BGRI, accessed on 10-12-2016).

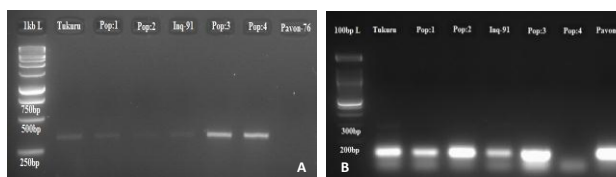
All the populations and parents showed Hope type allele of *Sr2* indicated by the production of three fragments after restriction digestion with *Bsp*HI (Fig. 2). This gene is fixed in the population and can easily be maintained in the segregating generations by utilizing morphological marker of pseudo-black chaff. Presence of *Sr2* in Pavon-76 has previously been reported by Njau *et al.* (2012).

**Authentication of *Yr17/Lr37/Sr38***

The genes for rust resistance, *Lr37*, *Sr38* and *Yr17* found in a fragment of *Triticum ventricosum* (Tausch) Cess. chromosome 2NS are trans-located to *Triticum aestivum* chromosome 2AS (Helguera *et al.*, 2003). All the parents and the populations illustrated the absence of 2NS translocations and hence *Lr37/Yr17/Sr38* genes (Fig. 2). The genes can be introduced into Inqilab-91 by exploiting appropriate sources. Though, virulence for *Lr37* and *Yr17* have been known to occur in various countries (Robert *et al.*, 1999) including Rawalakot and Azad Kashmir where moderate to complete susceptibility has been observed in isogenic lines (personal observation) but these genes are still resistant to a broad range of races and is effective when combined with other genes of rust resistance (Helguera *et al.*, 2003).



**Fig. 3:** Yellow rust severity in BC<sub>1</sub>F<sub>2</sub> populations compared to parents (Lowest Yr reaction shown by populations is used)



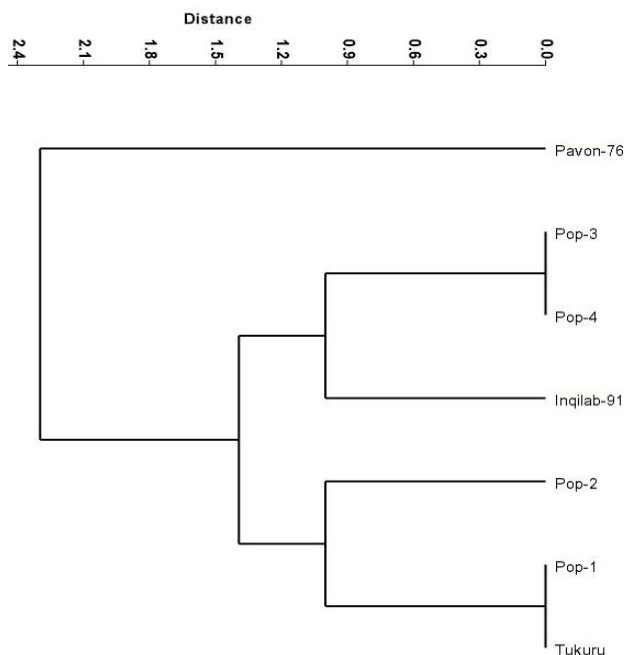
**Fig. 4:** PCR products for seedling resistance gene in parents and BC<sub>1</sub>F<sub>2</sub> populations of wheat (A. *Lr21* gene and B. *Lr39* gene)

### Field Response against Yellow Rust

Since the individual plants of back crossed F<sub>2</sub> populations were segregating hence the yellow rust response data indicated a variable reaction under field conditions. The populations who had *Lr46/Yr29* gene exhibited more yellow rust severity compared to ones having *Lr34/Yr18*. The plants in population 1 showed 30-60 MRMS reaction while those in population 2 showed 20-40 MRMS reaction (Fig. 3). When compared to susceptible parent Inqilab-91 (90S) the population 1 and 2 having *Lr34/Yr18* indicated 33-67% and 56-78% increase in yellow rust resistance respectively, while the population 3 and 4 having *Lr46/Yr29* indicated 22-44% increase in yellow rust resistance. This huge increase in resistance might be the result of possible transfer of some minor genes along with the slow rusting genes. The presence of 3 or 4 minor genes in addition to non-race specific genes has already been reported in both the parents Tukur and Poven-76 (Singh *et al.*, 2001).

### Screening of Populations and Parents for Race-Specific Genes

Resistance conferred by race specific genes is short lived and quickly overcome by the pathogen in a wide spread cultivation of cultivar carrying that gene; however these genes could be employed in combination with other resistance genes specifically the slow rusting genes.



**Fig. 5:** Tree diagram based on non-race specific and race specific genes for rust resistance

The back crossed F<sub>2</sub> populations and their parents were screened with the help of molecular markers of race-specific genes for rust resistance, so that the effectiveness of the resistance in the populations could be predicted and the inheritance of genes from the parents to the populations could be traced.

Presence/absence matrix for major genes displayed in Table 2 indicated that the genes *Lr32*, *Lr39* and *Lr51* were present in all the populations and parents except Pavon-76 while *SrCad* was found only in Pavon-76. The gene *Lr50* was missing in population 2.

Tree diagram based on non-race specific and race specific genes indicated that Population 3, 4 and population 1 and Tukur were similar (Fig. 5). Phenotyping also indicated a close relationship among these populations by showing almost similar kind of infection type.

### Conclusion

Based on molecular data the segregants of population 2 comprising *Lr34/Yr18*, *Sr2*, *Lr32*, *Lr39* and *Lr51* genes were the most appropriate ones to be exploited in succeeding generations as *Lr34/Yr18* was in homozygous form. The population 2 was back crossed once with the moderately resistant Tukur, highlighted the significance of back cross to secure the gene of interest in the succeeding generation. Crossing Inqilab-91 with resistant parent resulted in low infection type populations. Slow rusting genes from succeeding generations of population 2 and 3 and/or 4 could be combined in a single genotype through convergent crossing. Inclusion of more slow rusting genes

like *Lr37/Yr17/Sr38* in Inqilab-91 back ground might be more fruitful in producing durable resistance having a low infection type.

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