Materials and Methods
3. MATERIALS AND METHODS

3.1. Heterosis and Combining ability analysis

3.1.1. Plant materials

Seven popular Indian hexaploid wheat cultivars namely, HD 2385, HD 2428, HD 2643, HW 2031, HW 2055, HW 2061 and UP 262 were used as ovule parents (Lines). Four popular hexaploid wheat cultivars namely, HW 2044, HW 2004, UP 2338 and HW 2024 were used as pollen parents (Testers).

3.1.2. Crossing block and field layout

Seven ovule parents (Lines) and four pollen parents (Testers) were raised in a crossing block. The lines and testers were sown in single rows of 3m length with the rows and plants spaced at 30cm and 10cm, respectively, were raised. Crosses were made in all possible 28 combinations. Seeds of the 28 F₁ hybrids were planted along with their 11 parents in Randomised Block Design with five replications. The following data recorded: days to flowering, days to maturity, plant height (cm), number of tillers per plant, spike length (cm), number of spikelets per spike, grain yield per plant, 1000-grain weight per plant (g) and harvest index (%). The data were analysed according to Griffing (1956) as per requirement of a Line X Tester analyses.

3.1.3. Data recording

Five plants were randomly selected from each replication of all the crosses (F₁'s) and parents. Observations were recorded on individual plants for 10 quantitative and 5 qualitative characters and were measured using standard procedures and formulas as given below.

(I) Quantitative Characters

(i) Days to flowering - The total days from sowing to flowering of 50% of the main tillers were considered as days to flowering.
(ii) **Days to maturity** - The total days from sowing to maturity of 50% of the ears were recorded.

((iii) **Plant height (cm)** - Plant height was measured as the length from the soil surface to the apex of the five main tillers (excluding awns) at maturity.

(iv) **Number of tillers per plant** - Average number of tillers per plant was calculated for a total of ten plants in the middle row of each replication.

(v) **Spike length (cm)** - Length of ten main spikes (excluding awns) were recorded at maturity and averaged.

(vi) **Number of spikelets per spike** - Number of spikelets of each of ten spikes were counted and averaged.

(vii) **Grain yield per plant (g)** – Average grain yield per plant was calculated using ten plants in each replication.

(viii) **1000-grain weight (g)** - The average weight of 1000-grains from each of ten plants of each replication was determined.

(ix) **Biological yield (g)** – The total dry matter of a plant excluding the roots was determined for ten plants of each replication and averaged.

(x) **Harvest Index (%)**

\[
HI (%) = \frac{\text{Grain yield (g)}}{\text{Biological yield (g)}} \times 100
\]

II. **Qualitative Characters**

For qualitative characters, protein content, sedimentation value, pelshenke value, DBC (Dye Binding Capacity) value and lysine content were determined for each replication and averaged. The estimations of the above parameters were done according to AOAC (1996) and AACC (2000).
3.1.4. Statistical procedures

(i) Analysis of variance

The data were analysed as a randomized block design as suggested by Panse and Sukhatme (1967), partitioning the total variance into that due to replications and treatments for all the quantitative and qualitative characters. The following table shows the expected mean square values and the appropriate degrees of freedom in each case.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Mean square</th>
<th>Expected values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replications</td>
<td>(r-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatments</td>
<td>(t-1)</td>
<td>$M_t$</td>
<td>$\sigma^2e + r \sigma^2g$</td>
</tr>
<tr>
<td>Error</td>
<td>(r-1)(t-1)</td>
<td>$M_e$</td>
<td>$\sigma^2e$</td>
</tr>
<tr>
<td>Total</td>
<td>(r-1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where,  
- $r$ = Number of replications
- $t$ = Number of treatments
- $M_t$ = Mean square of treatments
- $M_e$ = Mean square of Error

'F' tests were done to test for significance of the main affects.

3.1.5. Estimation of heterosis

The overall mean value for each parent or hybrid over the five replications for each character was used for the estimation of heterosis. Heterosis was calculated as the percent deviation of mean of the $F_1$ hybrid from the mid parental value ($M_p$). Heterobeltiosis was estimated as the difference between the mean of the $F_1$ and that of the better parent for each character. Standard heterosis was estimated as the difference between the mean of the $F_1$ and that of the standard variety.

\[(i) \quad \text{Heterosis (d$_i$): Deviation of hybrid from mid parent} \% = \frac{F_1-\text{MP}}{\text{MP}} \times 100\]
(ii) Heterobeltiosis (dii): Deviation of hybrid from better parent (%) \[ F_{1}-BP \times 100 \]

(iii) Standard heterosis (diii): Deviation of hybrid from the standard variety (%) \[ F_{1}-SV \times 100 \]

Where,

(Mp) = Mid-parental value—For each character studied, the arithmetic mean of the two parents involved in each cross combination was taken as mid-parental value.

(Bp) = Better parent value—For each character studied, the mean of the superior parent in each cross was taken as the better parental value.

(Sv) = Standard variety value—For each character studied, the mean of the standard variety was taken as the standard varietal value.

\[ F_1 = \text{Mean of the hybrid (average of the measurements taken on 10 random plants in each of the 5 replications )}. \]

The significance of heterosis values was calculated using a ‘t’ test with reference to the CD (critical difference) values. (Wynne et al., 1970).

3.1.6. Combining ability analysis

The observations recorded for the hybrids were subjected to line x tester analysis and the general combining abilities (gca) of parents and specific combining abilities (sca) in the different crosses were calculated. The design of this experiment was according to the method developed by Kempthorne (1957) based on Experiment – II of Comstock and Robinson (1952). The model used to estimate the gca and sca effects of the ijk\textsuperscript{th} observation was:

\[ X_{ijk} = \mu + g_i + g_j + s_{ij} + e_{ijk} \]

Where,

\( \mu \) = population mean

\( g_i \) = gca effect of i\textsuperscript{th} ovule parent

\( g_j \) = gca effect of j\textsuperscript{th} pollen parent
sij = sca effect of ijth combination

eijk = error associated with ijkth observation

i = ith ovule parent

j = jth pollen parent, and

k = kth replication

(A) Analysis of variance for combining ability

The sums of squares due to gca and sca were worked out as follows:

SS due to gca (parents) = \frac{\text{**P}^2_{ii}}{r} - \text{C.F. (parents)}

SS due to sca (hybrids) = \frac{\text{**P}^2_{ii}}{r} - \text{C.F. (crosses)}

SS due to ovule parents = \frac{\text{**P}^2_{ii}}{r X m} - \text{C.F. (ovule parents)}

SS due to pollen parents = \frac{\text{**P}^2_{ii}}{r X f} - \text{C.F. (pollen parents)}

SS due to ovule parents = S.S. (crosses) - S.S. (ovule and pollen parents) - S.S. (pollen parents)

Where, Pii = the observation for ith parent

Pij = the observation for i x jth cross

r = number of replications

f = number of ovule parents

m = number of pollen parents

C.F. = correction factor

Analysis of variance for combining ability and expected mean squares.

The following table was set up for this purpose:
<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>MSS</th>
<th>Expectation of mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parents (gca)</td>
<td>(f+m)-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrids (sca)</td>
<td>(fm-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovule parents</td>
<td>(f-1)</td>
<td>(M_1)</td>
<td>(\alpha^2 e + r(Cov.(F.S.)-2Cov.(H.S.)) + mr Cov. (H.S.))</td>
</tr>
<tr>
<td>Pollen parents</td>
<td>(m-1)</td>
<td>(M_2)</td>
<td>(\alpha^2 e + r (Cov. (F.S.)- 2 Cov. (H.S.) + fr Cov. (H.S.))</td>
</tr>
<tr>
<td>Ovule x pollen Parents</td>
<td>(f-1) (m-1)</td>
<td>(M_3)</td>
<td>(\alpha^2 e + r (Cov.(F.S.)- 2 Cov. (H.S.))</td>
</tr>
<tr>
<td>Error</td>
<td>(f +fm -1) (r-1)</td>
<td>(M_4)</td>
<td>(\alpha^2 e)</td>
</tr>
</tbody>
</table>

Where,

- \(f = \text{number of ovule parents}\)
- \(m = \text{number of pollen parents}\)
- \(r = \text{number of replications}\)
- \(\alpha^2 e = \text{error mean square}\)

\[
\text{Covariance of half sibs} = \frac{(M_1-M_3) + (M_2-M_3)}{r(f + m)}
\]

\[
\text{Covariance of full sibs} = \frac{(M_1-M_4) + (M_2-M_4) + (M_3-M_4) + 6r \text{ Cov. H.S.} - r (f + m) \text{ Cov. H.S.}}{3r}
\]

Variance due to general combining ability (gca) and variance due to specific combining ability (sca) were derived as follows:

\[gca = \text{Covariance of H.S., and}\]

\[sca = \text{Covariance of F.S. - 2 Covariance of H.S.}\]
(B) Estimation of \textit{gca} and \textit{sca} effects

The individual effects were estimated as indicated below:

\[ X \ldots \]

i) \[ \mu = \frac{X}{m.f.f} \]

where,

\[ X \ldots = \text{Total of all the hybrid combinations.} \]

\[ X_{i} \ldots \]

\[ gi = \frac{X_{i} \ldots}{m.r \times m.f.r} \]

where,

\[ X_{i} \ldots = \text{Total of } i^{th} \text{ ovule parent summed over all the pollen parents and replications.} \]

\[ X_{j} \ldots \]

\[ gj = \frac{X_{j} \ldots}{f.r \times m.f.r} \]

where,

\[ X_{j} \ldots = \text{Total of } j^{th} \text{ pollen parent summed over all the ovule parents and replications.} \]

\[ X_{i} \ldots X_{j} \ldots \]

\[ sij = \frac{X_{i} \ldots X_{j} \ldots}{r \times m.r \times f.r \times m.f.r} \]

where,

\[ X(ij) = \text{Total of hybrid between the } i^{th} \text{ ovule parent and } j^{th} \text{ pollen parent summed over replications.} \]

The standard errors pertaining to \textit{gca} effects of ovule parents and pollen parents and \textit{sca} effects of different combinations were calculated as indicated below:
S.E. \((gi)\) ovule parents = \[
\frac{\text{Error variance}}{r.m}
\]

S.E. \((gj)\) pollen parents = \[
\frac{\text{Error variance}}{r.f}
\]

S.E. \((sij)\) ovule x pollen parents = \[
\frac{\text{Error variance}}{r}
\]

To test the differences of any two similar estimates, the square root of the product of the ‘t’ values against error degrees of freedom and standard error of differences were used as critical difference. The standard error was obtained as described below:

S.E. \((gi - gk)\) female = \[
\frac{2 \text{ Error variance}}{r.m}
\]

S.E. \((gj - gk)\) male = \[
\frac{2 \text{ Error variance}}{r.f}
\]

S.E. \((sij - skl)\) = \[
\frac{2 \text{ Error variance}}{r}
\]

(C) Estimation of genetic parameters

The genotypic variance, phenotypic variance, genotypic coefficient of variability, phenotypic coefficient of variability, heritability in the broad sense and genetic advance were calculated according to Singh and Choudhary (1979).

\[Mt - Me\]

i) Genotypic variance \((\sigma^2g)\) = \[
\frac{Mt - Me}{r}
\]

Where,

\[Mt = \text{Mean sum of squares for treatments}\]
Me = Error mean sum of squares
r = Number of replications

ii) Phenotypic variance ($\sigma^2_p$) = $\sigma^2_g + \sigma^2_e$

Where,
$\sigma^2_g = \text{Genotypic variance}$
$\sigma^2_e = \text{Error mean sum of squares}$

iii) Genotypic coefficient of variability (GCV)

$$\text{GCV} = \frac{\sqrt{\sigma^2_g}}{\text{X}} \times 100$$

Where,
$\sqrt{\sigma^2_g} = \text{Genotypic variance}$
X = Grand mean

iv) Phenotypic coefficient of variability (PCV)

$$\text{PCV} = \frac{\sqrt{\sigma^2_p}}{\text{X}} \times 100$$

Where,
$\sqrt{\sigma^2_p} = \text{Phenotypic variance}$
X = Grand mean

v) Heritability ($h^2$)

$$h^2 \text{ (broad sense)} = \frac{\sigma^2_g}{\sigma^2_p}$$

Where,
$\sigma^2_g = \text{Genotypic variance}$
$\sigma^2_p = \text{Phenotypic variance}$
vi) Genetic advance (GA)

\[
\text{Genetic advance (GA)} = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}} \times K
\]

Where, \( K = 2.06 \), the selection differential at 5 percent selection intensity.

GA as % of the overall mean = (GA / Grand mean) x 100

The estimated values of GCV and PCV, heritability and genetic advance as percent of the overall mean were classified as follows:

<table>
<thead>
<tr>
<th>Genetic parameter</th>
<th>Low</th>
<th>Moderate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCV and PCV</td>
<td>0 –10%</td>
<td>10-20%</td>
<td>20% and above</td>
</tr>
<tr>
<td>Heritability</td>
<td>0 - 30%</td>
<td>30-60%</td>
<td>60% and above</td>
</tr>
<tr>
<td>GA as % of mean</td>
<td>0 - 10%</td>
<td>10-20%</td>
<td>20% and above</td>
</tr>
</tbody>
</table>

(D) Correlation coefficients

Genotypic and phenotypic correlation coefficients between yield and yield components were worked out as suggested by Goulden (1959) and Singh and Choudhary (1979). The variance and covariance components were utilized to calculate the correlation coefficient by applying the following formula.

Phenotypic correlation coefficients:

\[
r_{p.1.2} = \frac{\text{Phenotypic covariance between characteristics 1 and 2}}{(\text{Phenotypic variance 1} \times \text{Phenotypic variance 2})^{1/2}}
\]

Genotypic correlation coefficients:

\[
r_{g.1.2} = \frac{\text{Genotypic covariance between characteristics 1 and 2}}{(\text{Genotypic variance 1} \times \text{Genotypic variance 2})^{1/2}}
\]
(E) Path coefficients

Path coefficient analysis was done for yield and yield components to study out the direct and indirect effects of different yield components on wheat grain yield. By using the genotypic correlation coefficients, path coefficients were worked out following the procedure suggested by Dewey and Lu (1959) and Singh and Choudhary (1979). Path coefficients were rated as indicated below following the scales suggested by Lenka and Misra (1973).

<table>
<thead>
<tr>
<th>SI No.</th>
<th>Path coefficient value</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>More than 1.00</td>
<td>Very high</td>
</tr>
<tr>
<td>2</td>
<td>0.30 to 0.99</td>
<td>High</td>
</tr>
<tr>
<td>3</td>
<td>0.20 to 0.29</td>
<td>Moderate</td>
</tr>
<tr>
<td>4</td>
<td>0.10 to 0.19</td>
<td>Low</td>
</tr>
<tr>
<td>5</td>
<td>0.00 to 0.09</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

3.2. Backcross breeding for rust resistance in hexaploid wheat

3.2.1. Details of seed materials

Four popular Indian hexaploid wheat cultivars namely, HD 2385, HD 2428, HW 2055 and UP 262, susceptible to one or all the three wheat rusts were used as the recipient parents. Nine donor parents contributed a total of seven leaf rust resistance genes (Lr19, Lr24, Lr26, Lr28, Lr32, Lr37, and Lr45), eight stem rust resistance genes (Sr24, Sr25, Sr31, Sr32, Sr34, Sr36, Sr38, and Sr44) and three stripe rust resistance genes (Yr8, Yr9, and Yr17) which occurred either singly or in closed linked clusters. The details of the recurrent and donor parents are listed in Table 1.

3.2.2. Place of study

The experimental work was conducted at the Indian Agricultural Research Institute (IARI), Regional station, Wellington, The Nilgiris, Tamilnadu, South India. Wellington (altitude 1850m above MSL, latitude 77° N) is a 'hot spot' for the three wheat rusts (leaf, stem, stripe), powdery mildew and eye spot disease.
### Table 1. List of hexaploid wheat lines and varieties used as parents in the backcross programme

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Wheat lines and cultivars used</th>
<th>Resistance genes</th>
<th>Alien donor species</th>
<th>Source of seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Wheat cultivars used as recurrent parents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>HD 2385</td>
<td>Lr23+</td>
<td>-</td>
<td>I.A.R.I., Regional Station, Wellington, The Nilgiris</td>
</tr>
<tr>
<td>2.</td>
<td>HD 2428</td>
<td>Lr23+, Lr1+, Sr7a + Sr11+, Yr2+ (KS)</td>
<td>-</td>
<td>I.A.R.I., Regional Station, Wellington, The Nilgiris</td>
</tr>
<tr>
<td>3.</td>
<td>HW 2055</td>
<td>Lr9</td>
<td>-</td>
<td>I.A.R.I., Regional Station, Wellington, The Nilgiris</td>
</tr>
<tr>
<td>4.</td>
<td>UP 262</td>
<td>Lr23 + Lr34+, Sr11+, Yr2+, Yr18+</td>
<td>-</td>
<td>I.A.R.I., Regional Station, Wellington, The Nilgiris</td>
</tr>
<tr>
<td>II. Hexaploid wheat stocks with alien gene(s) used as donor plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Cook*6/C 80-1</td>
<td>Lr19 + Sr25 + Sr36</td>
<td>Agropyron elongatum</td>
<td>Dr.R.A.McIntosh Australia</td>
</tr>
<tr>
<td>2.</td>
<td>Darf*6/3Ag/Kite</td>
<td>Lr24 + Sr24</td>
<td>Agropyron elongatum</td>
<td>I.A.R.I., Regional Station, Wellington, The Nilgiris</td>
</tr>
<tr>
<td>3.</td>
<td>Veery 'S'</td>
<td>Lr26 + Sr31 + Yr9</td>
<td>Secale cereale</td>
<td>CIMMYT, Mexico</td>
</tr>
<tr>
<td>4.</td>
<td>CS 2A / 2M / 4/2</td>
<td>Lr28 + Sr34 + Yr8</td>
<td>Aegilops speltoides</td>
<td>Dr.R.A.McIntosh Australia</td>
</tr>
<tr>
<td>6.</td>
<td>Thatcher *8/VPM 1 (RL 6081)</td>
<td>Lr37 + Sr38 + Yr17</td>
<td>Triticum ventricosa</td>
<td>Dr.S.M.S. Tomar, New Delhi</td>
</tr>
<tr>
<td>7.</td>
<td>RL 6144 ST-1</td>
<td>Lr45</td>
<td>Secale cereale</td>
<td>Dr.K.A.Kolmer Canada</td>
</tr>
<tr>
<td>8.</td>
<td>W 3531</td>
<td>Sr32</td>
<td>Aegilops speltoides</td>
<td>Dr.R.A.McIntosh Australia</td>
</tr>
<tr>
<td>9.</td>
<td>W 3521</td>
<td>Sr44</td>
<td>Agropyron intermedium</td>
<td>Dr.S.M.S. Tomar, New Delhi</td>
</tr>
</tbody>
</table>
The three wheat rusts perpetuate throughout the year, making it possible to screen material for rust resistance at any time of the year. Agro-climatic conditions at Wellington makes it possible to grow wheat throughout the year.

3.2.3. Methods used for backcross breeding

The rust resistance genes were transferred to Indian wheats by simple backcrossing (Crossing scheme). The 16 of the 18 rust resistance genes were dominant, while two (\(Lr26, Yr17\)) were recessive under Wellington conditions.

3.2.4. Screening for rust resistant seedlings / adult plants

Screening of seedlings and adult plants for resistance against stem, leaf and stripe rust was carried out either in a glasshouse or in the field following standard procedures as described by Joshi et al. (1982). Individual isolates or mixtures of rust races (Table 2) were used for resistance screening. The seedling reactions to rusts were scored as described by Nayar et al. (1994) (Table 3). Rust infection on the adult plants was recorded according to Peterson et al. (1948) (Table 4).

In the hybrids / derivatives that carry tightly linked rust resistance gene complexes for different type of rusts, for example, \(Lr19 + Sr25 + Sr36, Lr24 + Sr24, Lr26 + Sr31 + Yr9, Lr28 + Sr34 + Yr8, Lr37 + Sr38 + Yr17\), screening for rust resistance was done for one of the rusts.

3.2.5. Production and handling of backcross generations

The F\(_1\) hybrids, were backcrossed to the respective recurrent parents to produce the BC\(_1\). In this cross the recurrent parents were used as males. Screening for rust resistance was done in the BC\(_1\) both at the seedling stage in glasshouse (using part of the BC\(_1\) seed) and at the adult plant stage in the field (using the remaining part of BC\(_1\) seed). Inoculum mixtures of rust races were sprayed to screen for the resistance seedlings and plants. Resistant seedlings from the glasshouse were later transplanted into field. Resistant BC\(_1\) hybrids were further backcrossed to produce BC\(_2\), BC\(_3\), BC\(_4\) and BC\(_5\) hybrids,
CROSSING SCHEME

Crossing Scheme adopted for transfer of dominant rust resistance genes from hexaploid wheat stocks to hexaploid Indian wheats through backcross method.

Recipient
Indian wheat
parent (aa)
(2n=42)

X

Donor wheat
parent (AA)
(2n=42)

F₁ X aa

BC₁ X aa

BC₂ X aa

BC₂F₂

BC₂F₃

BC₂F₄

BC₂F₅

BC₃X aa

BC₄X aa

BC₅

BC₅F₂

BC₅F₃

BC₅F₄

BC₅F₅

@ - Selfing  * - Screening for rust resistance
Table 2. Rust races used for screening rust resistant seedlings/plants.

<table>
<thead>
<tr>
<th>Name of the rust</th>
<th>Rust races / bio - types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf rust (brown rust)</td>
<td>12-2, 77-1, 77-5, 77A, 77A-1, 104A</td>
</tr>
<tr>
<td>Stem (black rust)</td>
<td>11, 11A, 16, 34, 40-1, 40A, 117, 117A, 117A-1</td>
</tr>
<tr>
<td>Stripe rust (yellow rust)</td>
<td>20, 20A, 24, 38, 1</td>
</tr>
</tbody>
</table>
Table 3. Classification of seedling reaction types (Nayar et al. 1994) to wheat rusts.

<table>
<thead>
<tr>
<th>Reaction type / Grade</th>
<th>Category</th>
<th>Visible symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (naught)</td>
<td>Immune</td>
<td>No visible infection</td>
</tr>
<tr>
<td>0; (naught fleck)</td>
<td>Nearly immune</td>
<td>Slight necrosis / microflecking visible</td>
</tr>
<tr>
<td>; (fleck)</td>
<td>Very resistant</td>
<td>No uredia but hypersensitive flecks present</td>
</tr>
<tr>
<td>1 (one)</td>
<td>Resistant</td>
<td>Uredia minute, surrounded by distinct necrotic or necrotic border</td>
</tr>
<tr>
<td>2 (two)</td>
<td>Moderately resistant</td>
<td>Uredia small to medium, surrounded by chlorotic or necrotic border</td>
</tr>
<tr>
<td>3 (three)</td>
<td>Moderately Susceptible</td>
<td>Uredia medium in size, chlorotic areas may be present. In case of black rust coalescence infrequent, in yellow rust transverse banding is visible</td>
</tr>
<tr>
<td>33+ (Three three plus</td>
<td>Susceptible</td>
<td>In black rust uredia is large, coalescing, no necrosis but chlorosis may be present. In brown rust no chlorosis or necrosis; uredia profusely sporulating. In yellow rust uredia profusely sporulating.</td>
</tr>
<tr>
<td>3 + 4 (Three plus and four)</td>
<td>Highly susceptible</td>
<td>In black rust uredia is large, coalescing, no necrosis but chlorosis may be present. In brown rust no chlorosis or necrosis. Uredia profusely sporulating. In yellow rust uredia profusely sporulating and form stripes.</td>
</tr>
<tr>
<td>X</td>
<td>Heterogeneous / Mesothetic</td>
<td>Visible types of uredia</td>
</tr>
</tbody>
</table>

0 – 2 are resistant and 3 - X are susceptible
Table 4. Classification of adult plant reaction types (Peterson et al., 1948) to wheat rusts.

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>Visible symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (Free)</td>
<td>Free from rust infection</td>
</tr>
<tr>
<td>0 (Highly resistant)</td>
<td>Visible infection on plants</td>
</tr>
<tr>
<td>R (Resistant)</td>
<td>Visible chlorosis or necrosis, no uredia present</td>
</tr>
<tr>
<td>MR (Moderately resistant)</td>
<td>Small uredia are present and surrounded by either chlorotic or necrotic areas</td>
</tr>
<tr>
<td>M (Moderate)</td>
<td>Variable sized uredia are present some with chlorosis, necrosis or both</td>
</tr>
<tr>
<td>MS (Moderately susceptible)</td>
<td>Medium sized uredia are present and possibly surrounded by chlorotic area</td>
</tr>
<tr>
<td>S (Susceptible)</td>
<td>Large uredia are present, generally with little or no chlorosis or necrosis</td>
</tr>
<tr>
<td>T (Trace)</td>
<td>Severity is recorded as percentage. This recording process relies upon visual observations, and it is common to use the following intervals. Trace (T), 1, 10, 20, 30, 40, 60, 70, 80, 80, 90, 100 percent infection</td>
</tr>
</tbody>
</table>

0 - TR are resistant and TS – 100S are susceptible
respectively. In each generation, seedlings / adult plants were screened for rust resistance under both glasshouse and field conditions as. Some of the resistant BC<sub>2</sub> / BC<sub>3</sub> hybrids and all of the resistant BC<sub>5</sub> hybrids were selfed to obtain BC<sub>2F2</sub>, BC<sub>3F2</sub> and BC<sub>5F2</sub> populations, respectively.

### 3.2.6. Selection of plants showing good agronomic characteristics and assembly of near-isogenic lines (NILs)

In the BC<sub>2F2</sub>, BC<sub>3F2</sub> and BC<sub>5F2</sub>, the segregating populations were fairly large. At this stage 3-6 segregates showing reasonably good agrotype were selected. Seeds of the selected plants were sown to raise BC<sub>2F3</sub>, BC<sub>3F3</sub> and BC<sub>5F3</sub> populations. This process was continued until the F<sub>5</sub> when one line for each of the BC<sub>2F5</sub>, BC<sub>3F5</sub> and BC<sub>5F5</sub> was constituted for each cross combination. Rust resistant lines that were not agronomically acceptable were excluded from further evaluation.

### 3.2.7. Screening of the near-isogenic lines against individual races of the three wheat rusts

The near-isogenic lines (BC<sub>2F5</sub>, BC<sub>3F5</sub> and BC<sub>5F5</sub>) obtained from the different cross combinations were tested both at the seedling stage (glasshouse) and as adult plants in the field. Seedlings were inoculated with individual rust races, while the field inoculations were done with mixtures of rust races. NILs derived from the donor parents carrying the linked gene complexes, Lr<sub>19</sub> + Sr<sub>25</sub> + Sr<sub>36</sub>, Lr<sub>24</sub> + Sr<sub>24</sub>, Lr<sub>37</sub> + Sr<sub>38</sub> + Yr<sub>17</sub> were screened separately for each type of rust.

### 3.2.8. Evaluation of the near-isogenic lines for yield and other agronomic characters

Each near-isogenic line was grown in the field in a 3 x 2 metre plot, replicated three times. Recommended agronomic practices were followed. Six agronomically important characters viz., plant height (cm), tiller number per plant, spike length(cm), number of spikelets per spike, grain yield per plant(g) and 1000-grain weight(g) were recorded from the middle two rows of all the NILs. The
values recorded for plant height, number of tillers per plant and grain yield per plant were the average of 20 randomly selected plants, while the values for spikelet number and spike length were the average of 20 randomly selected spikes from each NIL. Thousand grain weight was determined using the bulked seed of each NIL.

All the agronomic characters of the NILs were compared with those of respective recurrent parents under rust free conditions. Rust free conditions were created by spraying with the effective systemic fungicide ‘Tilt’ (propiconazole 0.5%), which controls all three types of rust (Brahma et al., 1991). The first application of this chemical was done at the time of initial appearance of the rust, followed by three more applications at intervals of 15 days. A Factorial RBD was used to compare the means of populations, treatments and interactions.

Grain yields of the NILs were also compared with those of the respective recurrent wheat parents under rust epidemic conditions. Recurrent parents and NILs were treated with fungicide ‘Tilt’ as described above. In this trial, absolute checks (without chemical spray) were maintained and were sprayed with water. Finally, the plot grain yield was recorded and expressed in quintal / hectare.

3.2.9. Final selection of the lines on the basis of agronomic performance and seed quality

The NILs were finally selected on the basis of agrotype, grain yield and seed quality (seed shape, size, colour and weight).

3.2.10. Confirmation of transfer of rust resistance genes to recurrent parents

(i) Confirmation through morphological markers

Co-inheritance of resistance and morphological markers of the donor parents such as awnless (Darf /3Ag*6/kite), lax spike (RL6081) and reduced yellow flow pigment (cook*6/C 80-1) were studied in either F₁ or backcross derivatives.
(ii) **Confirmation through genetical studies**

Rust resistant plants of selected NILs were crossed with the universal susceptible wheat variety, Agra Local, some of the F₁ hybrids were selfed to obtain F₂ seeds and the remaining F₁ hybrids were backcrossed to the respective recurrent parents to produce BC₁. The F₁, F₂ and BC₁ plants were evaluated for rust reaction, both under glasshouse and field conditions, using mixed inoculum. Segregation data were recorded and the χ² test was used to test the goodness of fit to the expected segregation ratios.

(iii) **Through rust resistance provided by linked genes**

Near-isogenic lines carrying more than one type of rust resistance gene (due to tight linkage between them), for example: Lr19 + Sr25 + Sr36, Lr24 + Sr24, Lr26 + Sr31 + Yr9, Lr28 + Sr34 + Yr8, Lr37 + Sr38 + Yr17 were screened for resistance to the relevant types of rust. Evaluation were done both under glasshouse and field conditions.

(iv) **Confirmation through monosomic analysis**

NILs were also cytologically confirmed. The complete set of 21 monosomic lines of Chinese Spring were crossed with rust resistant NIL plants. F₁ monosomic plants were identified in each cross and selfed to obtain F₂ progenies. The disomic (control cross), F₁ and F₂ families were raised and screened for adult plant rust resistance. The χ² test was applied in the F₂ to test goodness of fit to the expected ratio.

(v) **Confirmation through biochemical studies**

Seed of the recurrent parents and their respective near-isogenic lines were surface sterilized for ten minutes in an aqueous solution of 0.1% mercuric chloride after soaking them in double distilled water for two hours and then rinsed several times in sterile distilled water. Seeds were then sown in plastic pots containing soil and farmyard manure (3:1), watered and then placed in glasshouse.
Rust inoculum (leaf rust or stem rust or stripe rust) was multiplied on the universally susceptible wheat cultivar, Agra Local. Mixtures of rust races were used to confirm the effectiveness of the respective rust resistance genes. Twenty-five day-old plants were inoculated individually with the spores (15mg / 100 leaves) by uniformly pasting the spore mass collected earlier on water sprayed leaves using a glass rod. The pots were watered well and kept inside wooden framed glass chambers (60cm x 45cm x 45cm) maintained at high relative humidity inside a glasshouse. Plant leaves were sampled for analysis at 0hr (just before inoculation), 1 day, 2 days, 3 days, 5 days and 7 days (or at regular intervals in some cases) after inoculation. Uninoculated (healthy) plants were maintained separately in a glasshouse. Changes in the enzymatic activity of polyphenol oxidase, catalase, lipoxygenase, soluble protein, lipid content, peroxidase, total chlorophyll content, total free aminoacid content and respiration rate were studied in the recurrent parents and NILs. Duncan’s Multiple Range Test (DMRT) (Duncan, 1955) was applied, wherever necessary, to compare the mean values of parents and the corresponding near-isogenic lines.

(a) Estimation of polyphenol oxidase and catalase enzyme activity

For estimating polyphenol oxidase and catalase activities, 1gm of fresh leaf tissue samples were homogenized in 10ml of cold 0.1M phosphate buffer (pH 7.0) containing 1mM cysteine hydrochloride and 0.1% ascorbic acid using a chilled mortar and pestle with acid washed sand as an abrasive. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 4°C. The supernatant was used for enzyme activity assays. Polyphenol oxidase was assayed following the method of Taneja and Sachar (1974). During activity assay the reaction mixture contained 2.0ml of 1.0% catechol solution as substrate, 0.2ml of enzyme extract and 1.8ml of 0.05M phosphate buffer (pH 6.6) in a final volume of 4ml. Boiled enzyme extracts served as the control.

Catalase activity was assayed following the method of Beers and Sizer (1952). During activity assay, the reaction mixture contained 0.1ml of 0.2M tissue extract, 0.5ml of 0.2M sodium phosphate buffer (pH 7.6), 0.3ml of hydrogen
peroxide and 2.1 ml of distilled water in a final volume of 3 ml. The enzyme activity was expressed as the change in absorbance per min. per mg protein at 240 nm.

(b) **Estimation of lipoxygenase enzyme activity**

For assaying lipoxygenase activity, 1 gm of fresh leaf sample was homogenized in 5.0 ml of 0.2 M phosphate buffer (pH 6.8) containing 1.0% Triton X-100, using a chilled mortar and pestle and and the homogenate was centrifuged at 18000 g at 4°C for 3 min. The supernatant was used for enzyme activity assay. Stock solutions A and B of substrate, linoleic acid, were prepared according to the method of Ben Aziz et al., (1970). For enzyme assay, stock solution B was diluted to 10 times in 0.2 M borate buffer (pH 9.0) containing 0.25% Tween 20. The reaction mixture contained 2.5 ml of substrate solution and 0.01 ml of enzyme extract and the subsequent increases in absorbance were noted at 234 nm for half an hour.

(c) **Estimation of lipid content**

Lipids were extracted from 20 gm of leaf tissue according to Hoppe and Heitefuss (1974). The combined methanol chloroform layers were evaporated to dryness at 40°C, lipid residue was dissolved in chloroform and dried under vacuum after filtration through a sintered glass funnel (pore size G-3). Lipid content was determined gravimetrically by recording the dry mass lipid residue.

(d) **Estimation of soluble proteins**

For protein extraction, the leaves (1 g) were homogenized in 10 ml of extraction buffer (30 mM Tris-HCl buffer pH 8.7, 1 mM DTT, 1 mM ascorbic acid, 1 mM EDTA Na₂, 5 mM MgCl₂, 50 mM insoluble PVP). The homogenate was centrifuged twice at 15000 rpm for 30 minutes at 4°C. Supernatant was used to precipitate protein using four volumes of chilled acetone at −20°C for 3 hours. Proteins were pelleted by centrifugation at 15000 rpm for 30 minutes. The pellets were air dried and solubilized in 100 μl of Laemmli SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl (pH 6.8) and boiled for 4 minutes and used for protein separation by SDS-PAGE. The SDS-PAGE
gels (12.5%) were stained with Coomasie Brilliant Blue R250 and destained in 40% methanol and 10% acetic acid. Total soluble proteins were estimated by the dye binding method of Bradford (1976) using BSA as a standard.

(e) Estimation of peroxidase activity

For estimating peroxidase activity, 1gm of fresh leaf tissue samples were homogenized in 3ml of cold 0.1M Tris-Hcl buffer, pH 8.5 containing 0.5M Nacl and using a chilled mortar and pestle with acid washed sand as an abrasive. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 20000rpm for 20 minutes in a refrigerated centrifuge at 3°C. The supernatant was used for peroxidase enzyme activity assay with guaiacol as hydrogen donor following the procedure of Johnson and Cunningham (1972). During the activity assay, the reaction mixture contained 540µmoles phosphate buffer (pH 6.0), 5.4µ moles guaiacol, 2.7µmoles of \( \text{H}_2\text{O}_2 \) and 0.5µml appropriately diluted enzyme or distilled water, in a final volume of 2.7ml. The reaction was initiated by adding the \( \text{H}_2\text{O}_2 \). Hydrogen peroxide concentration was determined spectrophotometrically, assuming 250nm = 0.0252cm\(^{-1}\) mM\(^{-1}\). Activity was expressed as µmoles \( \text{H}_2\text{O}_2 \) consumed / min /ml of undiluted enzyme, using the reaction 60µM \( \text{H}_2\text{O}_2 \) = 0.4 A\( _{470nm} \) cm\(^{-1}\). For the reaction volume of 2.7ml, 1.0 A\( _{470nm} \) = 0.4µM \( \text{H}_2\text{O}_2 \). To determine alterations in multiple molecular forms of peroxidase, electrophoresis was done with 7% acrylamide gels using a Mini-protein II Electrophoresis cell. Gel staining was done with benzidine (1%) and \( \text{H}_2\text{O}_2 \).

(f) Estimation of ribonuclease and nuclease

For estimating the specific activities of ribonuclease I and combined ribonuclease II and nuclease, all cultivars and NILs were used. Surface sterilized seeds were grown in a glass house. After 75 days of germination in a glass house under controlled conditions, plants were inoculated with a mixture of rust races (leaf / stem /stripe). Sampling was done on the 10\(^{th}\) and 15\(^{th}\) days after inoculation. Two grams of fresh leaf sample of each inoculated plant were homogenized in 4ml sodium acetate buffer (pH 5.2) in a chilled mortar and
pestle. The homogenate was filtered and centrifuged at 12000rpm for 20 minutes at 0°C. Specific activities of Ribonuclease-I and combined Ribonuclease-II and Nuclease-I were estimated according to Sodek and Wright (1969). One unit of enzyme is defined as the quantity of enzyme catalyzing an increase in \( A_{260} \) of 1.0 in the standard condition of assay. The spectrophotometric readings were converted to standard units as described by Wilson (1975).

\[
\text{RNAse units/ml} = \frac{A_{260} \times (\text{ml assay solution} + \text{ml ppt reagent}) \times \text{dilution factor}}{(\text{ml enzyme solution assayed}) \times \text{min. of incubation}}
\]

(g) Estimation of nuclear DNA total free phenol and tannin

The nuclear DNA content of the recurrent wheat parents (susceptible to rusts) and the wheat lines that carry different rust resistance genes were estimated with the help of Vicker's M 85 scanning microdensitometer. Fresh and healthy root tips (1cm) were fixed for 2 hours in freshly prepared 4\% formaldehyde and washed in distilled water for 24h with frequent changes. Material was fixed in Carnoy's fluid (6:3:1) for 24h and washed in distilled water for 30 min. with two changes. Root tips were hydrolysed in 1N HCl for 1h, followed by washing them with distilled water. After brief drying, root tips were stained in Feulgen solution (pH 2.2) for one hour. Finally the root tips were washed in SO\(_2\) water for 30 min. with frequent changes and slides were prepared by squashing the tip region in a drop of glycerol. To measure the nuclear DNA content of individual lines of wheat and their derivatives, \textit{Allium cepa} was taken as a standard. By measuring the optical densities of both the standard and wheat lines, proportionality was used to find the amount of nuclear DNA in the wheat line. The following formula was used for this purpose.

\[
\frac{\text{Amount of DNA in standard}}{\text{Optical density of standard}} = \frac{\text{Amount of DNA in wheat cultivar / line}}{\text{Optical density of wheat cultivar / line}}
\]
(h) **Estimation of total free phenol, tannin and other biochemical parameters**

Total free phenols and tannins were estimated in recurrent parents and their near-isogenic lines using an extract from seed powder prepared in 1% HCl in methanol following the method of Maxon and Rooney (1972). Tannin content was estimated quantitatively by using the method of Burns (1971). Chlorophyll was extracted from fresh leaves with 80% (V/V) acetone and determined according to the method of Mackinney (1941). For the determination of free amino acid, freeze dried leaves were used. Free amino acids were successively extracted with cold methanol / chloroform / water 12 / 5 / 3 V/V and 70% (V/V) ethanol according to the method of Bieleski and Turner (1966). The final extract was acidified (HCl) to pH 4.5 and purified by means of cation exchange chromatography. The total free amino acids were quantified according to the method of Yemm and Cocking (1955). Free proline was extracted and determined according to the method of Bates et al. (1973). The respiration rate was determined manometrically with the aid of a Gilson respirometer. O₂ uptake was measured and CO₂ was absorbed by 10% KOH on a piece of filter paper in the center well.

(vi) **Confirmation through molecular markers**

Recurrent parents and NILs for the leaf rust resistance gene *Lr 45* and stripe rust resistance gene *Yr 17* were screened for using 11 RAPD primers for *Lr 45*, and one RAPD primer, OP-Y 15, for stripe rust resistance gene, *Yr 17* (Robert *et al.*, 1999, 2000a,b).