Chapter-3
MATERIALS AND METHODS

The details of the material used and the techniques adopted during the course of the investigation are described in this chapter.

3.1 Development of base material

The material used in the study included new ‘A’ lines, maintainer lines (B lines) and new R lines. These lines resulted from the breeding program handled at Indo-American Hybrid Seeds (India) Pvt. Ltd., Research station, Dharwad. The methodology involved in developing these lines is given in fig-1.

3.1.1 Development of new A lines

New potential *G. hirsutum* varietal lines were identified during 1998 and involved them in developing 10 male sterile lines (A lines). Among them four lines were selected for the study.

3.1.2 Development of new R lines

An approach of developing new R lines based on sterile cytoplasm was followed where in male sterile based productive hybrids were crossed with highly potential conventional hybrids. The objective was to introduce genetic variability with the help of pooling genes from number of such potential hybrids. Since this population was based on sterile cytoplasm it was possible to identify restorer plants and advance them in segregating generation. In the group of 25 such new R lines, eight lines were selected for the study.
Fig-1: Schematic diagram for development of genetic stocks for the present study

- A line (G. harknessii) x G. hirsutum (4 lines) Gene pool of Robust plant types (G. hirsutum maintainer lines)
- Gene pool of Restorer lines (G. hirsutum) CGMS based x Conventional HH hybrids HH hybrids

Development of base material

- Recurrent back cross method
- Multiple crosses
- Multiple crosses
- Selection in segregating generations
- Selection in segregating generations
- 8 new R lines (Based on sterile cytoplasm)

Material used in the study

- 4 new A lines of desired genotypes
- 4 new Robust B lines
- 4 new Robust -B lines

A x R hybrids (32 combinations)

B x R hybrids (32 combinations)
3.1.3 Development of Robust lines (Maintainer)

The cultivated hirsutum varieties don't have restorer genes for the sterile cytoplasm barrowed from *G. harknessii* and thus these turns out to be maintainer lines. In a program on developing potential genotypes separate populations of robust lines (maintainer) were selected for the study.

3.2 Development of crosses

The detailed plan of the groups of crosses utilized in this study is given below:

3.2.1 A x R crosses

Cross combinations of CGMS based hybrids (Line x Tester design), maintainer lines, restorer lines and one commercial check included in this group. Since it is not possible to record observations on A lines used in the study, the corresponding B versions of these lines were included in the study.

<table>
<thead>
<tr>
<th>Entries</th>
<th>Symbol</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 CGMS A-lines x 8 R-lines</td>
<td>A x R</td>
<td>32</td>
</tr>
<tr>
<td>4 Maintainer (B) versions of A lines</td>
<td>B</td>
<td>4</td>
</tr>
<tr>
<td>8 R-lines</td>
<td>R</td>
<td>8</td>
</tr>
<tr>
<td>Check (Bunny)</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>

3.2.2 B x R crosses

Group of crosses involving new potential maintainer and restorer lines (Line x Tester design), parental lines and commercial check hybrid included are;
3.3 Experimental locations

The present investigations were undertaken at Indo-American Hybrid Seeds (India) Pvt. Ltd., Research Stations, Dharwad and Ranebennur during the year 2003-04 and 2004-05. The details of experiments conducted in different environments are presented in table-1.

Table-1: Crop season and environments details.

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Location</th>
<th>Season</th>
<th>Environments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A x R group</td>
<td>Dharwad</td>
<td>Kharif, 2003</td>
<td>E-1</td>
</tr>
<tr>
<td>A x R group</td>
<td>Ranebennur</td>
<td>Kharif, 2003</td>
<td>E-2</td>
</tr>
<tr>
<td>A x R group</td>
<td>Dharwad</td>
<td>Kharif, 2004</td>
<td>E-3</td>
</tr>
<tr>
<td>A x R group</td>
<td>Ranebennur</td>
<td>Kharif, 2004</td>
<td>E-4</td>
</tr>
<tr>
<td>B x R group</td>
<td>Dharwad</td>
<td>Kharif, 2003</td>
<td>E-1</td>
</tr>
<tr>
<td>B x R group</td>
<td>Ranebennur</td>
<td>Kharif, 2003</td>
<td>E-2</td>
</tr>
<tr>
<td>B x R group</td>
<td>Dharwad</td>
<td>Kharif, 2004</td>
<td>E-3</td>
</tr>
<tr>
<td>B x R group</td>
<td>Ranebennur</td>
<td>Kharif, 2004</td>
<td>E-4</td>
</tr>
</tbody>
</table>

E - Environment

3.4 Experimental material

The set of A, B and R lines utilized in this study are as follows:

(A) Cytoplasmic genetic male sterile lines

1) H 3493  
2) H 3496  
3) H 3497  
4) H 3503

(B) *G. hirsutum* recombinant Robust-B lines

5) H 3020  
6) H 3038  
7) H 3070  
8) H 3086
(C) Recombinant Restorer lines with sterile cytoplasm (R- lines)

9) RH 89
10) RH 95
11) RH 118
12) RH 122
13) RH 157-1
14) RH 158
15) RH 173-5
16) RH 179-7

3.4.1 Cultural practices

The experimental material was planted on a medium black soil at Dharwad and red soil at Ranebennur locations under irrigated conditions. Entries of A x R and B x R group of crosses, parents along with check (Bunny) were sown at both the locations during kharif 2003-04 and 2004-05 in randomized block design with two replications. Plot size of each entry was two rows of 6 m length with a spacing of 90 cm between rows and 60 cm between the plants within a row. Recommended dose of 4-ton farmyard manure per acre was incorporated to the soil before sowing. At the time of sowing applied inorganic fertilizers 30: 30: 30 kg of N, P$_2$O$_5$ and K$_2$O per acre. After 30 and 45 days after sowing 30 kg / acre nitrogen was applied in two splits.

To keep the crop free of weeds three hand weeding and five times intercultivations were carried out. To control pests the crop was sprayed with Imidachloprid (0.25 ml) followed by Deltamethrin + Trizopos (1.5 ml), Endosulfan 35%EC (2.5 ml), Chloropyriphos (2.5 ml), Fenvalerate (1 ml), Indaxacarb (0.5 ml) and Profenophos 40% + Cypermethrin 4% (1 ml) dissolved per litre of water and applied when it was required. Irrigations were given to maintain adequate moisture in the soil for crop growth. Weather at these locations during the two years is presented in Appendix I.
3.5 Collection of data and recording of observations

Five plants in each entry were selected randomly and tagged. These tagged plants were used for recording observations on plant height, number of monopodia, number of sympodia, number of bolls per plant, boll weight (g), number of seeds per boll, seed index, lint index and ginning outturn (GOT%). Seed cotton yield and lint yield were obtained from entire plot and converted to per hectare. Days to 50 per cent flowering was recorded on number of plants per plot. The details of recording of observations are as follows.

3.5.1 Days to 50 per cent flowering

The number of days taken from the date of sowing to the opening of first flower in 50 per cent of the plants in each entry was recorded.

3.5.2 Plant height

Individual plant height in each entry was measured in centimeters at maturity from the base of the plant to tip of plant.

3.5.3 Number of monopodia per plant

The total number of monopodial branches present on the main stem of the individual plant was counted at final harvest and average was worked out.

3.5.4 Number of sympodia per plant

The total numbers of sympodial branches present on main stem of the individual plant were recorded at final harvest and average was worked out.

3.5.5 Number of bolls per plant

The total number of matured good open bolls picked till the end of last picking was recorded.
3.5.6 Boll weight

The weight of seed cotton obtained from randomly collected twenty bolls was recorded and the average boll weight was expressed in grams.

3.5.7 Number of seed per boll

Number of seeds per boll based on twenty randomly collected bolls was recorded.

3.5.8 Seed index

Seed index was taken as weight of 100 seeds and expressed in grams.

3.5.9 Lint index

Lint index represents the absolute weight of lint produced per seed and was computed as per the formula:

\[
\text{Lint index} = \frac{\text{Weight of lint} \times \text{seed index}}{\text{Weight of seeds}}
\]

3.5.10 Ginning outturn

Ginning outturn (GOT) is the ratio of weight of lint to that of seed cotton expressed in percentage as given below:

\[
\text{Ginning outturn} = \frac{\text{Weight of lint} \times 100}{\text{Weight of seed cotton}}
\]

3.5.11 Seed cotton yield

Weight of total seed cotton obtained from individual plot from all pickings was recorded and expressed in Kg/ha.

3.5.12 Lint yield

The lint yield Kg/ha was obtained from the following formula:
Materials and methods

3.5.13 Fiber quality analysis

Top five crosses in both A x R and B x R group along with check hybrid were subjected for fiber quality analysis. The following fibre quality parameters were measured using High Volume Instrument (HVI) at Central Institute for Research on Cotton Technology, Mumbai.

3.5.13.1 Fibre length (mm)

Fibre graph was used to measure the 2.5 per cent span length (mm) that is defined as the distance spanned by a specified percentage of fibres in the specimen being tested.

3.5.13.2 Uniformity Ratio

Uniformity ratio was calculated as the ratio of 50 percent span length to 2.5 percent span length and expressed as percentage.

\[
U R (\%) = \frac{50\%\ S.L. \times 100}{2.5\%\ S.L.}
\]

where, SL = Span length

3.5.13.3 Fibre fineness

The micronaire instrument that gives the measurement of resistance to flow of air by fibre plugs was taken an indirect measure of fibre fineness. The fineness expressed in microgram per inch or micronaire value.
3.5.13.4 Fibre maturity coefficient

Maturity coefficient represents the ratio of the mature, half-mature, and immature fibre in a sample of lint. This was determined with the help of a micronaire instrument by airflow method. A quantity of 3.24 g. of lint was fed to the instrument and compressed air was allowed to pass through one sample with and without spacer. From the difference between the two readings maturity coefficient was calculated.

3.5.13.5 Fibre strength

Stelometer was used to measure the fibre strength at 3.2 mm gauge strength determination. Fibre strength was expressed in gram per tex and calculated as

\[
\text{Tenacity (g/tex)} = \frac{\text{Breaking strength (kg) } \times 15.0}{\text{Weight of bundle (mg)}}
\]

3.5.13.6 Strength / Length ratio (S/L ratio)

The fibre strength and fibre length values were utilized to calculate ratio of strength to length which indicates for every millimeter of length what is the strength (g/tex) put up by genotype.

\[
\text{S / L ratio} = \frac{\text{Fibre strength}}{\text{Fibre length}}
\]

3.6 Statistical analysis

The data collected in respect of four environments were subjected to the following analysis

a. Analysis of variance for each environment separately and pooled analysis of variance.

b. Heterosis and Combining ability analysis.
3.6.1 Layout of the experiment

At each environment the Line x Tester crosses of A x R and B x R group of hybrids their parents were sown in adjoining blocks and randomization was done within the groups. The observations mentioned in section 3.5.1 to 3.5.12 were subjected for Line x Tester analysis using computer programme SPAR1.

3.6.2 Environment wise analysis of variance

Means based on data collected on the individual plants were computed. The analysis of variance for each of the characters observed in each environment was made following Panse and Sukhatme (1961).

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>Expected MSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replications</td>
<td>(r-1)</td>
<td>-</td>
</tr>
<tr>
<td>Treatment</td>
<td>(t-1)</td>
<td>$\sigma^2e + r \sigma^2t$</td>
</tr>
<tr>
<td>Error</td>
<td>(r-1)(t-1)</td>
<td>$\sigma^2e$</td>
</tr>
</tbody>
</table>

3.6.3 Pooled analysis of variance

The analysis of variance was carried out by following method of Al-Jibouri et al. (1958).

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>MSS</th>
<th>Expected MSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replications in environments</td>
<td>e(r-1)</td>
<td>MSS1</td>
<td>$\sigma^2e + r \sigma^2g \times e + r e \sigma^2g$</td>
</tr>
<tr>
<td>Environments</td>
<td>(e-1)</td>
<td>MSS2</td>
<td>$\sigma^2e + r \sigma^2g \times e$</td>
</tr>
<tr>
<td>Varieties</td>
<td>(t-1)</td>
<td>MS2</td>
<td>$\sigma^2e + r \sigma^2g \times e$</td>
</tr>
<tr>
<td>Varieties x Environment</td>
<td>(t-1) (e-1)</td>
<td>MS3</td>
<td>$\sigma^2e$</td>
</tr>
<tr>
<td>Error</td>
<td>e(r-1) (t-1)</td>
<td></td>
<td>$\sigma^2e$</td>
</tr>
</tbody>
</table>
3.6.3.1 Testing the significance of variety x environment interaction

The error mean square MS3 was used to test the significance of variety x environment interaction. The 'F' ratio given by MS2/MS3 was compared with table value at (t-1) (e-1) d.f. against (t-1) e (r-1) d.f.

3.6.4 Combining ability analysis

Combining ability analysis was done following Line x Tester approach developed by Kempthorne (1957) and emphasized by Arunachalam (1974) for estimating the combining ability effects and combining ability variances. The ANOVA for combining ability is given below. Spar-1 statistical programme was used for analysis of pooled and environment wise combining ability.

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>MSS</th>
<th>Expected MSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>(r-1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Crosses</td>
<td>(mf-1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lines</td>
<td>(f-1)</td>
<td>ML</td>
<td>σ²e + rσ²lt + rτσ²l</td>
</tr>
<tr>
<td>Testers</td>
<td>(m-1)</td>
<td>MT</td>
<td>σ²e + rσ²lt + rlσ²t</td>
</tr>
<tr>
<td>Line x Tester</td>
<td>(f-1) (m-1)</td>
<td>MLT</td>
<td>σ²e + rσ²lt</td>
</tr>
<tr>
<td>Error</td>
<td>(r-1) (mf-1)</td>
<td>ME</td>
<td>σ²e</td>
</tr>
</tbody>
</table>

The covariance of half sibs and full sibs were estimated using the expected mean sum of square as follows.

\[
\text{Co-variance of half sibs} = \frac{ML + MT - 2MLT}{r (m+f)}
\]

\[
\text{Co-variance of full sibs} = \frac{ML + MT + MLT - 3ME + 6 r \text{ cov (HS)} - r (m+f) \text{ cov (HS)}}{3 r}
\]

\[\sigma^2 \text{ gca} = \text{cov (HS)}\]

\[\sigma^2 \text{ sca} = \text{cov (FS)} - 2 \text{ cov (HS)}\]

\[\sigma^2 \text{ GCA of lines (females)} = \frac{ML - MLT}{r m}\]
Chapter 3

Materials and methods

\[ \sigma^2 \text{GCA of testers (males)} = \frac{ML - MLT}{rf} \]

\[ \sigma^2 \text{SCA of hybrids} = \frac{MLT - ME}{r} \]

Where, ML = Mean squares due to lines
MT = Mean squares due to testers
MLT = Mean squares due to line x tester
ME = Error mean sum of square.

3.6.4.1 Estimation of general and specific combining ability effects

The model used to estimate general combining ability (gca) and specific combining ability (sca) effects was,

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

Where, \( \mu \) = Population mean
\( g_i \) = gca effect of \( i^{th} \) female parent (line)
\( g_j \) = gca effect of \( j^{th} \) male parent (tester)
\( S_{ij} \) = sca effect of \( ij^{th} \) combination
\( e_{ijk} \) = Error associated with \( ij^{th} \) cross in \( k^{th} \) replication

\( i \) = Number of female parents
\( j \) = Number of male parents
\( k \) = Number of replications

3.6.4.2 General combining ability effects

(a) Lines:

\[ g_i \text{ (gca effect of } i^{th} \text{ line)} = \frac{x_{i..}}{mr} - \frac{x_{...}}{mfr} \]

Where, \( x_{i..} \) = Total of \( i^{th} \) female parent over all male parents and replications
(b) Testers:

\[ g_j (\text{gca effect of } j^{th} \text{ tester}) = \frac{x_{j.}}{fr} - \frac{x_{...}}{mfr} \]

where, \( x_{...} \) = Total of all hybrids over all the replications

\( x_{j.} \) = Total of \( j^{th} \) male parent over all the female parents and replications.

3.6.4.3 Pooled score for gca effects

3.6.4.3.1 Simple pooled gca score method

In this approach, significant gca effect in desirable direction is given positive weightage (+1) and negative weightage (-1) is given for gca effect in undesirable direction (Arunachalam and Bandopadhyay, 1979). These values are added over different yield influencing characters to arrive at pooled score of gca effects.

3.6.4.3.2 Per cent gca method

The raw gca values have to be converted into per cent gca values, the minute differences in gca values are also focused and the possible problem arising out of the differences in unit of measurement, high and lower per se gca values associated with the type of character concerned are overcome (Mallikarjun, 2005).

3.6.4.3.3 Weighted per cent gca method

The experience of the breeders would suggest sometimes that, in arriving the pooled score, it is desirable to attach differential weightages to each of the characters studied depending upon its economic importance,
contribution to yield etc. These weightages can be multiplied with per cent gca values of corresponding characters and then added to arrive at the pooled gca score for each parent (Mallikarjun, 2005).

### 3.6.4.4 Specific combining ability effects

\[ S_{ij} = \frac{x_{ij}}{r} - \frac{x_{..}}{mr} - \frac{x_{ij}}{fr} + \frac{x_{..}}{mfr} \]

where, \( S_{ij} \) = Specific combining ability effects of \( ij^{th} \) combination

\( X_{ij} \) = Total of \( ij^{th} \) combination over all the replications

The standard errors of estimates of gca and sca effects were estimated using the following formulae,

- Standard Error (SE) of \( g_i \)'s = \( \sqrt{\frac{ME}{mr}} \)
- Standard Error (SE) of \( g_j \)'s = \( \sqrt{\frac{ME}{fr}} \)
- Standard Error (SE) of \( S_{ij} \)'s = \( \sqrt{\frac{ME}{r}} \)

Where, ME, m, f and r take the same meaning as described earlier.

The estimates of \( g_i \)'s, \( g_j \)'s and \( S_{ij} \)'s were tested for their statistical significance by means of a 't' test.

\[ t_{gi} = \frac{g_1 \cdot 0}{SE (g_i)} ; \quad t_{gj} = \frac{g_1 \cdot 0}{SE (g_j)} ; \quad t_{sij} = \frac{S_{ij} \cdot 0}{SE (S_{ij})} \]

### 3.6.5 Heterosis studies

Per cent heterosis of \( F_1 \) over mid parent and check was calculated by the method of Turner (1953) and Hayes et al. (1955).

\[ \text{Per cent heterosis over Mid parent} = \frac{\text{Mean } F_1 - \text{Mid parent}}{\text{Mid parent}} \times 100 \]
Per cent heterosis over check = \( \frac{\text{Mean } F_1 - \text{Check}}{\text{Check}} \times 100 \)

### 3.6.5.1 Standard error of estimates

To compute the standard error (SE) of estimates for heterosis, mean square due to error (E) from RBD analysis was considered.

For testing heterosis over mid parent

\[
\text{SE (MP)} = \sqrt{\frac{3 \times E}{2r}}
\]

For testing heterosis over commercial check

\[
\text{SE (CC)} = \sqrt{\frac{2 \times E}{r}}
\]

The critical difference values in each case were worked out by multiplying their corresponding SE values with table ‘t’ value at error degrees of freedom at 5 and 1 per cent levels of significance.

### 3.6.6 Genotype-environment interaction

The magnitude of G x E interaction was assessed for each character and each genotype. This was worked out as per the procedure suggested by Eberhart and Russell (1966). The analysis of variance for stability as per this model is algebraically represented ANOVA Table below:
### Chapter-3 Materials and methods

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of Freedom</th>
<th>Sum of squares</th>
<th>Mean Squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>$nv - 1$</td>
<td>$\sum i \sum_j Y^2_{ij} - CF\ (TSS)$</td>
<td></td>
</tr>
<tr>
<td>Genotypes</td>
<td>$(v-1)$</td>
<td>$\sum i Y^2_{ij} / n - CF\ (GSS)$</td>
<td>$MS_1$</td>
</tr>
<tr>
<td>Genotype x Environment</td>
<td>$(v-1)\ (n-1)$</td>
<td>$TSS - GSS - ESS$</td>
<td>$MS_2$</td>
</tr>
<tr>
<td>Environment + (Genotype x Environment)</td>
<td>$v(n-1)$</td>
<td>$\sum i \sum_j Y^2_{ij} - \frac{\sum j Y^2_{ij}}{n}$</td>
<td>$MS_3$</td>
</tr>
<tr>
<td>Environment (linear)</td>
<td>1</td>
<td>$\frac{1}{v} \left( \sum_j Y_{ij}^2 / \sum j I^2_j \right)$</td>
<td>$MS_4$</td>
</tr>
<tr>
<td>G x E (linear)</td>
<td>$(v-1)$</td>
<td>$\sum i \frac{(\sum_j Y_{ij}^2 I_j)^2}{\sum j I^2_j}$ - Env. (linear ss)</td>
<td>$MS_5$</td>
</tr>
<tr>
<td>Pooled Deviation</td>
<td>$v(n-2)$</td>
<td>$\sum_j \sum_j \sigma^2_{ij}$</td>
<td>$MS_5$</td>
</tr>
<tr>
<td>Genotype I</td>
<td>$(n-2)$</td>
<td>$\sum_j Y^2_{ij} - \frac{(Y_i)^2}{n} - \frac{(\sum_j Y_{ij} I_j)^2}{\sum j I^2_j}$</td>
<td>$Md_1$</td>
</tr>
<tr>
<td>Genotype V</td>
<td>$(n-2)$</td>
<td>$\sum_j Y^2_{vj} - \frac{(Y_v)^2}{n} - \frac{(\sum_j Y_{vj} I_j)^2}{\sum j I^2_j - \sum \sigma^2_{vj}}$</td>
<td>$MS_5$</td>
</tr>
<tr>
<td>Pooled error</td>
<td>$n (r-1)\ (v-1)$</td>
<td>$\sum_i Y_{ij} - CF\ (TSS)$</td>
<td>$MS_5$</td>
</tr>
</tbody>
</table>

Where,

- $n$ = number of environments
- $r$ = number of replications
- $v$ = number of genotypes
- CF = correction factor

Various ‘F’ ratios calculated were,

i) To test the significance of the differences among the variety means:

$$F = \frac{MS_1}{MS_4}$$

ii) To test for the genetic differences among varieties for their regression on the environmental index.

$$F = \frac{MS_3}{MS_4}$$

iii) To test deviation from regression for each variety

$$F = \left[ \frac{\left( \sum_j \sigma^2_{ij} \right) / S-2}{MS_5} \right] / MS_5$$
In this model the total sum of squares (SS) has been partitioned into (i) SS due to genotype, (ii) SS due to environments and G x E (linear) and (iii) pooled error. Further, the sum of squares due to environment plus genotype x environment (linear) has been partitioned into (a) SS due to environments (linear), (b) SS due to genotype environment (linear) and (c) pooled deviation. Again the SS due to pooled deviation has been divided in to deviation from regression due to each (i^{th}) genotype (i=1,2,........v).

The regression co-efficient (bi) were examined for their deviation from unit value by employing 't' test.

\[ t = \frac{1 - bi}{SE \ bi} \quad ; \quad p=0.05 \text{ probability at } n-2 \text{ d.f.} \]

\[ SE \ bi = \left( \frac{\sum i \sigma^2 i / n-2}{\sum j i^2 j} \right)^{1/2} \]

A joint consideration of the three parameter i.e.,

i) The mean performance of the variety over environments x1,

ii) The regression co-efficient bi and

iii) The deviation from linear regression S^2di, is used to define stability of genotype, or in other words it gives an estimate of stability of the genotypes. The estimation of deviation from regression suggests the degree of reliance that could be put to linear regression in interpretation of the data. If these values are significantly deviating from zero, the expected phenotype cannot be predicted satisfactorily. When deviations are not significant, the conclusions may be drawn by joint consideration of mean yield and regression values (Finlay and Wilkinson, 1963 and Eberhart and Russell, 1966) as below:
### Materials and methods

<table>
<thead>
<tr>
<th>Regression</th>
<th>Stability</th>
<th>Mean yield</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b=1$</td>
<td>Average</td>
<td>High</td>
<td>Well adapted to all environments</td>
</tr>
<tr>
<td>$b=1$</td>
<td>Average</td>
<td>Low</td>
<td>Poorly adapted to all environments</td>
</tr>
<tr>
<td>$b&gt;1$</td>
<td>Below average</td>
<td>High</td>
<td>Specifically adapted to favourable environments</td>
</tr>
<tr>
<td>$b&lt;1$</td>
<td>Above average</td>
<td>High</td>
<td>Specifically adapted to unfavourable environments</td>
</tr>
</tbody>
</table>

Regression value of unity is interpreted as average stability since the average slope over all varieties on the environmental index will be unity.

#### 3.6.7 Stability analysis

Stability analysis was carried out as per Eberhart and Russell (1966).

The model employed is presented below:

$$ Y_{ij} = \mu_i + b_i i + \epsilon_{ij} $$

Where, $Y_{ij}$ = The mean of $i^{th}$ genotype at $j^{th}$ environments.

$\mu_i$ = The mean of $i^{th}$ environments.

$b_i$ = The regression co-efficient of the $i^{th}$ genotype on the environmental index which measures the response of the $i^{th}$ genotype of varying environments.

$i_j$ = The environmental index obtained as the deviation of mean of all genotypes at the $j^{th}$ environments from the grand mean.

$\sigma_{ij}$ = Deviation from the regression of the $i^{th}$ genotype at $j^{th}$ environment.

These parameters were estimated as follows

1) $\mu = \bar{Y}_i / n$

2) $b_i = \sum j Y_{ij} i / \sum j i^2_j$

3) $S^2 di = \sum j (\sigma^2 ij / n-2) S^2 e/r$
Where, \( n \) = Number of environments.

\[ Y_i = \text{Total of genotype } i \text{ over all the environments.} \]

\[ Y_{ij} = \text{Performance of } i^{\text{th}} \text{ genotypes at } j^{\text{th}} \text{ environment.} \]

\[ S^2 e / r = \text{Estimated pooled error or the variance of a genotype mean at the } j^{\text{th}} \text{ environment.} \]

iv) \( I_j = \text{Environmental index} \)

\[
I_j = \frac{(Y_{..}) - (Y_{.j})}{n} \frac{v}{v_n}
\]

\[ Y_{..} = \text{Grand total of all genotypes over all environments.} \]

\[ Y_{.j} = \text{Total of all genotype at } j^{\text{th}} \text{ location.} \]

\[ v = \text{Number of genotypes.} \]

v) \( \sum S^2 ij = \text{Sum of squares of deviations from the regression line obtained as} \)

\[
\sum jY^2 ij = \frac{(Y^2..)}{n} - \frac{(\sum j Y_{ij} I_j)^2}{\sum j I^2 j}
\]

Stability analysis of 64 hybrids representing the two groups of crosses along with check hybrid (65) was carried out based on data obtained from four environments.

### 3.7 Development of suitable in vitro pollen germination media (PGM)

The literature survey indicated that, number of culture media and methods have been tried for the in vitro germination and growth. Burke et al. (2004) modified the media described by Taylor (1972) and Wauford (1979) and achieved a very high level of pollen germination. Although they have simplified the media requirement by eliminating the need for some salts and hormones, the stringent microenvironment (temperature, humidity, pH & Carbon source) was recommended to enhance pollen tube growth.
Maintenance of stringent microenvironment conditions during pollen germination would be difficult possibility. The reported media was not suitable for the selected genotypes and failed to get uniformly high level of pollen germination. Therefore several experiments were conducted to standardize the media for in vitro pollen germination of G. hirsutum genotypes. The basic media (BM) composition of Burke et al. (2004) is 10% (w/v) Agarose, 25% (w/v) Sucrose, 0.52 mM KNO₃, 3.06 mM MNSO₄, 1.66 mM H₃BO₃, 0.42 mM MgSO₄ and 1.0 μM A₃ Gibberellic acid. To this basic media agarose of different concentrations was added. Agarose concentrations varied from 0 to 4% are as shown in the table-2.

Table-2: Basic media with different concentration of agarose.

<table>
<thead>
<tr>
<th>PGM</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGM-1</td>
<td>BM</td>
</tr>
<tr>
<td>PGM-2</td>
<td>BM+ 0% Agarose</td>
</tr>
<tr>
<td>PGM-3</td>
<td>BM+ 0.2% Agarose</td>
</tr>
<tr>
<td>PGM-4</td>
<td>BM+ 1% Agarose</td>
</tr>
<tr>
<td>PGM-5</td>
<td>BM+ 2% Agarose</td>
</tr>
<tr>
<td>PGM-6</td>
<td>BM+ 3% Agarose</td>
</tr>
<tr>
<td>PGM-7</td>
<td>BM+ 4% Agarose</td>
</tr>
</tbody>
</table>

BM: Basic Media, PGM: Pollen Germination Media

3.7.1 Pollen germination and tube growth

Flower buds, which will be ready to open in the next day, were selected on previous evening. They were kept in petridish laid with moist blotting paper on the both the sides (-RH 70-80%). Next day morning the dehiscing anthers were selected for pollen grain germination studies.

The sitting drop method in cavity slides was used for pollen germination. For each cavity about 100 μl of media was dispersed. The pollen grains from dehiscing anthers were sprinkled on the media uniformly.
For each PGM and genotypes five cavities were prepared. Seven PGM (Table-2) were selected and two genotypes were tested. The pollen grains were incubated after 90 minutes at room temperature in cavity slides with moist blotting paper (~RH 70-80%). Ten fields per cavity were randomly chosen for recording observations on pollen germination and tube growth under projection microscope. Pollen grain with a tube length equal to its diameter was taken as germinated and the pollen tube length was measured on the screen of projection microscope. Each division was considered as one unit. The germination and tube growth was increased from 0.2% to 1.0% agarose in both the genotypes. Highest germination and tube length growth was observed in basic media + 1% agarose. Hence, PGM-4 was taken as standard basic pollen germination media (PGM) for 16 G. hirsutum genotypes in the present study.

Standardized media was used to evaluate pollen growth parameters of 16 parental lines (8 maintainer and 8 restorers). Four cavities per genotype per replication with two replications were adapted. For each cavity five fields were randomly selected for recording observations. The experiment was repeated twice to confirm the results.

3.8 RAPD Marker studies

Two hybrids (H3497 x RH179-9 and H3086 x RH173-5) along with their parents were subjected for RAPD analysis for determining the genetic relationship. The individual plant DNA from each line was analyzed with RAPD primers. The laboratory work was done at Biotechnology division of Indo-American Hybrid Seeds (India) Pvt. Ltd., Bangalore.
3.8.1 DNA extraction

The DNA was extracted from the selected hybrids and their parents by following CTAB (cetyltrimethylammonium bromide) extraction method (Lodhi et al., 1994).

1. Solutions

- Extraction buffer: 20 mM sodium EDTA and 100 mM Tris-HCl, adjusted pH to 8.0 with HCl, added 1.4 M NaCl and 2.0% (w/v) CTAB.

Dissolved CTAB by heating to 60°C and stored at 37°C then 0.2 % of β-mercaptoethanol added just before use.

- Chloroform : octanol 24:1 (v/v)
- 5 M NaCl
- TE buffer: 10 mM Tris-HCl and 1 mM EDTA, adjusted pH to 8.0 and autoclaved
- RNAase A (Sigma R9009: 10 mg/mL)

2. Collected 0.5 g of unexpanded young leaves and ground to fine powder using liquid nitrogen.

3. Added 5 ml of extraction buffer to the ground leaves and mixed in the mortar.

4. Poured the slurry into clean 15 ml polypropylene centrifuge tubes (Laboratory Product Sales, Rochester, New York; LX 4109), by rinsing the mortar and pestle with 1 ml of extraction buffer and added to the original extract.

5. After adding 50 mg polyvinylpolypyrrolidone (PVP) (Sigma, P6755) and inverted the tubes several times to mix thoroughly with the leaf slurry (100 mg PVP/g leaf tissue).

6. Incubated at 60°C for 25 minutes and cooled to room temperature.
7. 6 ml of chloroform:octanol was added and mixed gently by inverting the tubes 20 to 25 times to form an emulsion.

8. The solution was centrifuged at 6000 rpm for 15 minutes at room temperature.

9. Transferred the top aqueous phase to a new 15 ml centrifuge tube with a wide-bore pipette tip. A second chloroform:octanol extraction step was repeated.

10. Then added 0.5 volume of 5M NaCl to the aqueous solution recovered from the previous step and mixed well.

11. Two volumes of cold (-20°C) 95% ethanol was added and refrigerated (4 to 6°C) for 15-20 minutes until DNA strands begin to appear.

12. Differential spinning at 3000 rpm for three minutes and then increased speed to 5000 rpm for an additional three minutes at room temperature. This step helped to keep DNA at the bottom of the centrifuge tube.

13. The supernatant solution was dispensed and DNA pellet was removed and washed with cold (0 to 4°C) 76% ethanol. Completely removed ethanol without drying the DNA pellet by leaving the tubes uncovered at 37°C for 20 to 30 minutes.

14. Dissolved in 200 to 300 µL TE.

15. Treated with 1 µL RNAase A per 100 µL DNA solution and incubate at 37°C for 15 minutes.

16. Quantification of DNA was done in a spectrophotometer at A260.

17. DNA was stored at -20°C.
3.8.2 RAPD-PCR amplification

**Template DNA:** Genomic DNA of hybrids and their parents were used as template DNA per reaction.

**Random primers:** Commercial kits of A, F, G and AM series of random decamer primers obtained from Operon Technologies Inc. USA.

Twenty-four decamer primers viz., OPA series (5 primers), OPF series (14 primers), OPG series (4 primers) and OPAM (1 primer) were used to screen the hybrids and their parents.

**Taq DNA polymerase:** 10x Taq assay buffer and dNTPs were obtained from M/s. Bangalore Genei Pvt. Ltd., Bangalore.

**Chemicals:** Analytical grade chemicals were obtained locally.

**Thermal cycler:** Primus-96 plus

**Stock solutions**

- 100 μM Random Primer
- 25 ng μl⁻¹ Template DNA
- 3.0 u μl⁻¹ Taq DNA polymerase

**Master mix for PCR (25 μl tube⁻¹)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq 10x assay buffer</td>
<td>2.50 μl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.50 μl</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>1.00 μl</td>
</tr>
<tr>
<td>Primer (100μM)</td>
<td>2.50 μl</td>
</tr>
<tr>
<td>Template DNA (25 ng μl⁻¹)</td>
<td>2.00 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>13.50 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase (3.0u μl⁻¹)</td>
<td>2.00 μl</td>
</tr>
<tr>
<td>Total</td>
<td>25.00 μl</td>
</tr>
</tbody>
</table>

One primer at a time was used to screen for DNA polymorphism by RAPD-PCR assay with DNA from different genotypes of cotton as template.

Master mix required for a set of 6 reactions was prepared fresh, from the
original stocks. The master mix was distributed (23 μl tube\(^{-1}\)) to 6 tubes containing 2.0 μl each of the template DNA from different genotypes and the mixture was given a short spin to mix the contents.

**Thermal cycling**

- Sterilized micro tubes were numbered from 1 to 6
- 2.0 μl of template DNA from individual genotypes was added to each tube
- 23 μl of master mix was added to all the tubes and was given a short spin to mix the contents.
- The tubes were placed in to the thermal cycler for 45 cycles of PCR, the programme consisted of three distinct steps as follows

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature and Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C for 25 seconds</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>35°C for 45 seconds</td>
</tr>
<tr>
<td>Primer extension</td>
<td>72°C for 1 minute</td>
</tr>
</tbody>
</table>

An initial denaturation step of 3 minutes at 94°C and a final synthesis step of 15 minute at 72°C were also included. After completion of 45 cycles, samples were held at 4°C, in the thermal cycler, until the contents were loaded on to the gels for electrophoresis.

**3.8.3 Separation of amplification products by Agarose gel electrophoresis**

Agarose (2.9 g) was added to a conical flask containing 200 ml of 1x TBE buffer and it was melted by heating the solution on a gas stove and the solution was stirred to ensure even mixing and complete dissolution of agarose. The solution was then cooled to about 40-45°C and 2 to 3 drops of
ethidium bromide (0.5 µg ml⁻¹) was added. The solution was poured into the gel-casting platform after inserting the comb in the trough, while pouring sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1x TBE) so as to cover the wells completely.

The amplified products (25 µl) to be analyzed were carefully loaded into the sample wells, after adding 2-3 µl of Ficol containing Bromophenol blue with the help of a micropipette. Electrophoresis was carried out at 200-volt hours, until the tracking dye migrated to the end of the gel. The gel was taken out from electrophoretic apparatus and stained by placing it in distilled water containing ethidium bromide (0.5 µg ml⁻¹) for 10 minutes. Ethidium bromide stained DNA bands were viewed under UV transilluminator and image was captured.

3.8.4 Analysis of amplification profiles

Amplification profiles of 24 primers were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The data for 24 primers, which produced scorable bands, was used for further computations.

\[
\text{Per cent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100
\]