CHAPTER-III

MATERIALS AND METHODS

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CHAPTER-III
MATERIALS AND METHODS

The field experiment was conducted at Regional Agricultural Research Station (RARS), Akbarpur, Karimganj during sali (kharif) season in 2005 and Field Trial Station (FTS), Mahakal, Karimganj during two sali seasons in 2006 and 2007.

3.1. EXPERIMENTAL MATERIALS:

The experimental material consisted of 49 genotypes including 47 local bold grained rice genotypes and two high yielding genotypes i.e. Monohar Sali and Ranjit. Most of the local genotypes were collected from farmer’s fields of Cachar, Karimganj and Hailakandi districts and some of them were also collected from sali germplasm collection of RARS, Akbarpur, Karimganj. The list of genotypes is presented in the Table 3.1.

Table No. 3.1 Name of the genotypes included in the experiment

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Name</th>
<th>Genotype</th>
<th>Name</th>
<th>Genotype</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>G_1</td>
<td>Soulpona</td>
<td>G_18</td>
<td>Baodun</td>
<td>G_35</td>
<td>Haladhar Sali</td>
</tr>
<tr>
<td>G_2</td>
<td>Lati Sali</td>
<td>G_19</td>
<td>Kapilee Dhan</td>
<td>G_36</td>
<td>Chatri Sail</td>
</tr>
<tr>
<td>G_3</td>
<td>Chuto Mula</td>
<td>G_20</td>
<td>Chandmoni</td>
<td>G_37</td>
<td>Maghi Sali</td>
</tr>
<tr>
<td>G_4</td>
<td>Kartic Kalma</td>
<td>G_21</td>
<td>Gouarchor</td>
<td>G_38</td>
<td>Mala</td>
</tr>
<tr>
<td>G_5</td>
<td>Basanta Bahar</td>
<td>G_22</td>
<td>George Sail</td>
<td>G_39</td>
<td>Dome Sail</td>
</tr>
<tr>
<td>G_6</td>
<td>Karmi Sail</td>
<td>G_23</td>
<td>Herapowa</td>
<td>G_40</td>
<td>Agani Sali</td>
</tr>
<tr>
<td>G_7</td>
<td>Soularpona</td>
<td>G_24</td>
<td>Dudh Mula</td>
<td>G_41</td>
<td>Rashi</td>
</tr>
<tr>
<td>G_8</td>
<td>Chaku Sail</td>
<td>G_25</td>
<td>Gajep Sali</td>
<td>G_42</td>
<td>Hathi Sali</td>
</tr>
<tr>
<td>G_9 ✓</td>
<td>Dhola Mula</td>
<td>G_26</td>
<td>Kali Makuri</td>
<td>G_43</td>
<td>Shem Sali</td>
</tr>
<tr>
<td>G_10</td>
<td>Kuiari Sali</td>
<td>G_27</td>
<td>Bata Sail</td>
<td>G_44</td>
<td>Daura Sali</td>
</tr>
<tr>
<td>G_11 ✓</td>
<td>Matonga</td>
<td>G_28</td>
<td>Bar Madhava</td>
<td>G_45</td>
<td>Chingra Sali</td>
</tr>
<tr>
<td>G_12</td>
<td>Chafa Sail</td>
<td>G_29 ✓</td>
<td>Monohar Sali</td>
<td>G_46 ✓</td>
<td>Khasi Dhan</td>
</tr>
<tr>
<td>G_13</td>
<td>Methi Chikon</td>
<td>G_30</td>
<td>Ranjit</td>
<td>G_47 ✓</td>
<td>Malati</td>
</tr>
<tr>
<td>G_14</td>
<td>Probat Jeera</td>
<td>G_31</td>
<td>Hacha Lath</td>
<td>G_48 ✓</td>
<td>Zoli</td>
</tr>
<tr>
<td>G_15</td>
<td>Kamal Bhog</td>
<td>G_32</td>
<td>Samras</td>
<td>G_49 ✓</td>
<td>Lalita Sali</td>
</tr>
<tr>
<td>G_16</td>
<td>Betguti Dhan</td>
<td>G_33 ✓</td>
<td>Atha Sail</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G_17</td>
<td>Heera Dhan</td>
<td>G_34 ✓</td>
<td>Latha Sail</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2. METHODS:

3.2.1. Environment:

The genotypes were grown in *sali* (kharif) season in 2005, 2006 and 2007.

3.2.2. Meteorological Data:

The meteorological data on rainfall, temperature, relative humidity and bright sunshine hours were recorded for the *sali* season extending over June to December. The data are presented in appendix-I to III.

3.2.3. Design of experiment and layout:

The experiment was laid out in the RARS field situated at 25° 9’ N latitude and 92° 16’ E longitude. The design of the experiment was randomized block design (RBD) with three replications for each genotype. The size of each plot was 8m x 1m with a crop geometry of 20cm x 20cm. The soil pH was around 5.4.

3.2.4. Time of application of fertilizer:

Well rotten FYM @ 10 tons/ha was applied during field preparation along with N:P:K @ 20:10:10 in the form of urea (in two split doses), single super phosphate and muriate of potash respectively. Half of urea, full dose of single super phosphate and muriate of potash were applied as basal at the time of final puddling. The remaining half dose of urea was applied at the time of panicle initiation stage as top dressing.

3.2.5. Nursery bed and transplanting of seedlings:

Healthy seeds were selected by putting them in water and stirring well, the floated seeds were rejected. The germinated seeds were sown in the raised seedbed. The seedlings were transplanted on the main field after 28 days. Only one seedling was transplanted per hill.
3.2.6. Plant Protection Measure:

As a prophylactic measure against Hispa, Carbofuran 3G was applied @ 30kg/ha. However, no serious incidence of insect pests and diseases was observed during the period of experiment.

3.2.7. Collection of Data:

For the collection of field data, ten plants were randomly selected from the middle rows leaving the border rows in each plot. The data were recorded on individual plant basis from ten randomly selected plants and the mean value was calculated for further analysis.

3.3. Quantitative Characters:

3.3.1 Days to 50 percent flowering (DF): It was recorded as the number of days from the date of sowing seeds to the date when 50 percent of the plants in a plot showed half emergence of panicle from the leaf sheath.

3.3.2 Plant height (PH): The actual measurement was recorded in cm from the soil surface to the tip of the tallest panicle excluding awns at maturity.

3.3.3 Flag leaf length (FLL): Flag leaf length was measured in cm from the base of the collar to the tip of the blade after heading.

3.3.4 Flag leaf breadth (FLD): Flag leaf breadth was recorded in cm from the broadest part of the leaf blade after heading.

3.3.5 Effective branch tillers per hill (EBT/hill): The actual number of panicle bearing tillers per hill was recorded as EBT/hill.

3.3.6 Panicle length (PL): The panicle length was measured in cm from the base to the tip of the panicle excluding awn at maturity.

3.3.7 Grains per panicle (Gr/P): All the filled and empty grains were recorded in the sample panicle.
3.3.8 Sterility percentage (SP): The ratio of total chaffy grains (empty grains) to total grains per sample panicle was calculated and expressed in percentage.

3.3.9 Grain length (Gr L): The mean length (mm) was recorded from the base of the lower most sterile lemma to the tip (apiculus) of the fertile lemma or palea by a screw gauge. In case of awned varieties, the grain length was measured to a point comparable to the tip of the apiculus. Mean of ten samples was recorded.

3.3.10 Grain breadth (Gr B): Grain breadth was measured as the distance across the fertile lemma and the palea at the widest point. Mean of ten samples was recorded.

3.3.11 Grain length: breadth ratio (L/B-ratio): From the average length and breadth of the grain, the ratio was calculated.

3.3.12 Kernel length (KL): The lengths of ten-sample grains after dehulling were measured by screw gauge in mm and the mean length was recorded.

3.3.13 Kernel breadth (KD): The total breadths of ten-sample grains after dehulling were measured in mm by a screw gauge and the mean breadth was recorded.

3.3.14 Kernel length/breadth ratio (L/B ratio): From the average length and breadth of kernel, the ratio was calculated.

3.3.15 1000-grain weight (Gr.wt): The weight in gram of 1000 healthy seeds of each sampled plant was recorded after proper drying.

3.3.16 Yield (YD): The grain yield of one square meter area was randomly selected from the plot. One square meter area accommodated 5x5 that is 25 hills at 20 cm x 20 cm spacing. Total grain yield of one square meter area was determined from 5 random spots in a plot and the mean weight was calculated. From the mean weight of 1 square meter area total grain yield per hectare was calculated.
### 3.4 Qualitative Characters:

The qualitative characters were recorded as per the manuals of IRRI and AAU. Stages of growth on which observations were recorded for specific character are given below:

<table>
<thead>
<tr>
<th>Code</th>
<th>Stages of Growth</th>
<th>Code</th>
<th>Stage of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Germination</td>
<td>6</td>
<td>Heading</td>
</tr>
<tr>
<td>2</td>
<td>Seedling</td>
<td>7</td>
<td>Milk stage</td>
</tr>
<tr>
<td>3</td>
<td>Tillering</td>
<td>8</td>
<td>Dough stage</td>
</tr>
<tr>
<td>4</td>
<td>Stem elongation</td>
<td>9</td>
<td>Mature grain</td>
</tr>
<tr>
<td>5</td>
<td>Booting</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 3.4.1 Leaf blade colour (LBC, at growth stage 4-6):

The leaf blade colour was observed during stem elongation, booting and heading stages and the number codes recorded were as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Types of observation</th>
<th>Code</th>
<th>Types of observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Light Green</td>
<td>4</td>
<td>Purple tips</td>
</tr>
<tr>
<td>2</td>
<td>Green</td>
<td>5</td>
<td>Purple margins</td>
</tr>
<tr>
<td>3</td>
<td>Dark green</td>
<td>6</td>
<td>Purple blotch</td>
</tr>
</tbody>
</table>

#### 3.4.2 Leaf blade pubescence (LBP):

The character was observed at growth stage 5 & 6 by rubbing fingers from the tip to bottom on the leaf. The presence of hair on the blade surface was classified as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glabrous</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>3</td>
<td>Pubescent</td>
</tr>
</tbody>
</table>

#### 3.4.3 Flag leaf angle:

The flag leaf angle was measured at growth stage 4 & 5 by determining the angle of attachment between the flag leaf blade and the main panicle axis. The classification and code were as follows:
1  Erect (30° or less)
2  Intermediate (45°)
3  Horizontal (90°)
4  Descending (More than 90°)

3.4.4 Sheath colour (SC): The sheath colour was observed during early vegetative stages (3 & 4). The colour was observed visually as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green</td>
</tr>
<tr>
<td>2</td>
<td>Purple lines</td>
</tr>
<tr>
<td>3</td>
<td>Light purple</td>
</tr>
<tr>
<td>4</td>
<td>Purple</td>
</tr>
</tbody>
</table>

3.4.5 Tillering ability (TA): It was counted. The code classification was as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Very high (more than 25 tillers/plant)</td>
</tr>
<tr>
<td>2</td>
<td>Good (20 to 25 tillers/plant)</td>
</tr>
<tr>
<td>3</td>
<td>Medium (10 to 19 tillers/plant)</td>
</tr>
<tr>
<td>4</td>
<td>Low (5 to 9 tillers/plant)</td>
</tr>
<tr>
<td>5</td>
<td>Very low (less than 5 tillers/plant)</td>
</tr>
</tbody>
</table>

3.4.6 Septum colour (Sep. C): The joint colour of nodes and internodes were observed visually.

3.4.7 Panicle type (Pn T): The panicles were classified according to their mode of branching, angle of primary branches and spikelet density at drought stage (8) as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Compact</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>3</td>
<td>Open</td>
</tr>
</tbody>
</table>
3.4.8 Panicle exertion (EXS): The inability of panicle to exert fully was commonly considered a genetic defect. Environmental and disease factors also contribute to such defect. The character was observed at milk (7), dough (8) and mature grain (9) stages and classified as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Well exerted</td>
</tr>
<tr>
<td>3</td>
<td>Moderately well exerted</td>
</tr>
<tr>
<td>5</td>
<td>Just exerted</td>
</tr>
<tr>
<td>7</td>
<td>Partly exerted</td>
</tr>
<tr>
<td>9</td>
<td>Enclosed</td>
</tr>
</tbody>
</table>

3.4.9 Panicle threshability (Thr.): The character was judged by firmly grasping the panicle and pulling the hand over it. The estimate of the percentage shatter grain reflected the threshability. The character was judged at mature grain stage as per classification given below:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Difficult (less than 1%)</td>
</tr>
<tr>
<td>3</td>
<td>Moderately difficult (1 to 5 %)</td>
</tr>
<tr>
<td>5</td>
<td>Intermediate (6 to 25%)</td>
</tr>
<tr>
<td>7</td>
<td>Loose (26 to 50%)</td>
</tr>
<tr>
<td>9</td>
<td>Easy (51 to 100%)</td>
</tr>
</tbody>
</table>

3.4.10 Apiculus colour (ApC): The character was recorded during growth stage 7 to 9 as per classification given below:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White</td>
</tr>
<tr>
<td>2</td>
<td>Straw</td>
</tr>
<tr>
<td>3</td>
<td>Brown (tawny)</td>
</tr>
<tr>
<td>4</td>
<td>Red</td>
</tr>
<tr>
<td>5</td>
<td>Red apex</td>
</tr>
<tr>
<td>6</td>
<td>Purple</td>
</tr>
<tr>
<td>7</td>
<td>Purple apex</td>
</tr>
</tbody>
</table>
3.4.11 Awning (An): The character was observed during mature grain (9) stage as per following classification:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>Short and partly awned</td>
</tr>
<tr>
<td>5</td>
<td>Short and fully awned</td>
</tr>
<tr>
<td>7</td>
<td>Long and partly awned</td>
</tr>
<tr>
<td>9</td>
<td>Long and fully awned</td>
</tr>
</tbody>
</table>

3.4.12 Kernel colour (KC): The kernel colour was observed in dehulled grains and was recorded as follows:

<table>
<thead>
<tr>
<th>Type of observation</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. White</td>
<td>wt</td>
</tr>
<tr>
<td>2. Off white</td>
<td>off. wt</td>
</tr>
<tr>
<td>3. Brownish white</td>
<td>Br. wt</td>
</tr>
<tr>
<td>4. Red</td>
<td>Rd</td>
</tr>
</tbody>
</table>

3.4.13 Endosperm type (EnT): The character was judged by the response of cut surface of endosperm to weaken KI-I solution (prepared in 10:1 ratio). Waxy starch endosperm stains brown, non-waxy endosperm stains blue black and classified as follows:

<table>
<thead>
<tr>
<th>Code</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non glutinous (non-waxy)</td>
</tr>
<tr>
<td>2</td>
<td>Glutinous (waxy)</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

3.5 Biochemical Characters:

3.5.1 Preparation of sample: 100g of mature seeds were oven dried at 40°C till a constant weight attained. The dried sample was grinded in an electric grinder and was stored in a desiccator for all the subsequent chemical analysis.
3.5.2 **Determination of crude protein content:** The crude protein content was determined by Micro-Kjeldahl’s method of protein estimation.

The nitrogen in protein of the sample was converted to ammonium sulphate \((\text{NH}_4)_2\text{SO}_4\) by sulphuric acid \((\text{H}_2\text{SO}_4)\) during digestion. This salt on steam distillation liberated ammonia and was collected in boric acid solution and titrated against standard acid \((0.1\text{N HCl})\). Since 1 ml of 0.1N acid was equivalent to 1.401mg nitrogen \((\text{N})\); calculation was made to arrive at the nitrogen content of the sample.

A measured quantity of the sample \((100\text{mg})\) was digested in a 30ml Pyrex digestion tube with 2g potassium sulphate \((\text{K}_2\text{SO}_4)\) and 90mg of mercuric oxide that acted as catalyst and 3ml of conc. \(\text{H}_2\text{SO}_4\). Boiling chips were added to the digestion flask and the sample was digested in the digestion chamber until the solution becomes colourless. After cooling the digest, it was diluted with a small quantity of distilled ammonia free water and transferred into the distillation apparatus. The Kjeldahl flask was then rinsed with successive small quantities of water. In a 100ml conical flask, 5ml of boric acid \((2\%)\) solution with a few drops of mixed indicator was taken with the tip of the condenser dipping below the surface of the solution. 10ml of sodium hydroxide – sodium thiosulphate solution was added to the test solution in the distillation flask.

The distillate i.e. liberated ammonia was collected on boric acid and at least 15 to 20ml of distillate was collected. The solution so obtained was titrated against HCl of known strength until the first appearance of violet colour, the end point. A reagent blank with an equal volume of distilled water was run simultaneously to know the volume of standard acid required for blank.
The nitrogen content of the sample was calculated based on the formula:

\[
\text{Total nitrogen (A)} = \frac{(a - b) \times \text{Normality of HCl} \times 14.01}{\text{Weight of the sample (g)}}
\]

Where, 
- \(a\) = volume of standard acid required for sample
- \(b\) = volume of standard acid required for the blank

The crude protein content in 100g of sample was calculated by multiplying total nitrogen (A) with conversion factor 5.95. 
\[i.e., \text{Crude protein content (g /100g of sample)} = A \times 5.95\]

3.5.3 Determination of Total Soluble Proteins: About 0.5 g of dried powder sample was taken in a centrifuge tube and to it, 5ml of 0.1N NaOH was added and stirred in cold condition for 15 minutes followed by centrifugation at 3000G for 10 minutes. The supernatant was decanted to a test tube and the residue was again treated with 5 ml of 0.1N NaOH and stirred for 15 minutes followed by centrifugation as before. The supernatant was decanted in the previous test tube. The procedure was repeated four times. 2 ml of the above supernatant was mixed with 2ml of 20% TCA, kept in cold for one hour for precipitation, and again centrifuged at 3000G for 10 minutes. The residue was dissolved in 10 ml of 0.1N NaOH. This solution was used for protein determination following Lowry's method.

3.5.4 Estimation of lysine content:

The protein in the grain sample was hydrolyzed with a proteolytic enzyme, papain. The \(\alpha\)-amino groups of the derived amino acids were made to form a complex with copper. The \(\epsilon\)-amino group of lysine would not couple with copper and made to form \(\epsilon\)-dinitropyridyl derivative of
lysine with 2-choro-3,5-dinitropyridine. The excess pyridine was removed with ethyl acetate and the colour of e-dinitropyridyl derivative was read at 390nm.

**Materials:**

a) Solution A: 2.89g CuCl₂·2H₂O in 100 ml of water
b) Solution B: 13.6g Na₃PO₄·12H₂O in 200 ml water
c) Sodium Borate Buffer 0.05 M, pH 9.0
d) Copper Phosphate Reagent: 100 ml of solution A was poured to 200 ml of solution B with constant swirling and centrifuged, the supernatant was discarded. The pellet was resuspended in 15 ml borate buffer and centrifuged. The supernatant was discarded and the process was repeated three times. After third washing, the pellet was resuspended in 80 ml of borate buffer. The reagent was prepared fresh during use.
e) 3% solution of 2-Chloro-3,5-Dinitropyridine in methanol. This solution was prepared fresh prior to use.
f) 0.05 M Sodium Carbonate buffer (pH 9.0)
g) Amino Acid Mixture: In a mortar 30 mg alanine, 50 mg glutamic acid, 60 mg aspartic acid, 20 mg cysteine, 300 mg glutamic acid, 40 mg glycine, 30 mg histidine, 30 mg isoleucine, 80 mg leucine, 30 mg methionine, 40 mg phenylalanine, 80 mg proline, 50 mg serine, 30 mg threonine, 30 mg tyrosine and 40 mg valine were grinded together and 100 mg of this mixture was dissolved in 10 ml of sodium carbonate buffer (0.05 M, pH 9.0)
h) 400 mg of technical grade papain (Sigma Co., USA) in 100 ml of 0.1 M sodium acetate buffer (pH 7.0)
i) 1.2 N HCl
j) Ethyl acetate
Procedure:

1) 5 ml of papain solution was added to 100 mg of defatted grain powder sample and incubated overnight at 65°C. It was then cooled to room temperature, centrifuged and the clear digest was decanted.

2) In 1 ml of digest in a centrifuged tube, 0.5 ml of carbonate buffer and 0.5 ml of copper phosphate suspension were added.

3) The mixture was shaken for 5 minutes in a vortex mixer and then centrifuged.

4) To 1 ml of supernatant 0.1 ml of pyridine reagent was added and mixed well by shaking for 2 hours.

5) 5 ml of 1.2 N HCl was added and mixed.

6) It was extracted three times with 5 ml of ethyl acetate and ethyl acetate (top) layer was discarded.

7) The absorbance of aqueous layer was read at 390 nm.

8) A blank was prepared with 5 ml of papain alone and the steps 1 to 7 were repeated.

9) 62.5 mg of lysine mono-hydrochloride was dissolved in 50 ml of carbonate buffer (1 mg lysine/ml). 0.2, 0.4, 0.6, 0.8 and 1 ml of the solution were pipetted out in separate test tube and the volume of each tube was made up to 1 ml with carbonate buffer. 4 ml of papain was added to each tube and mixed well. 1 ml from each tube was pipetted out and 0.5 ml of amino acid mixture was added followed by copper phosphate suspension. The steps 3 to 7 were carried out. The standard curve represented the values for 40, 80, 120, 160 and 200 µg lysine.

Calculation:

A standard curve was prepared from the reading of the standard lysine. The absorbance of the blank was subtracted from that of the sample and the lysine content of the sample was calculated from the graph.
3.5.5 Estimation of Tryptophan content:

The indole ring of tryptophan developed an orange-red colour with ferric chloride under strongly acidic condition. The colour intensity was measured at 545 nm.

Materials:

1) Papain solution: 400 mg of technical grade papain was dissolved in 100 ml of 0.1 N sodium acetate buffer pH 7.0. The solution was prepared fresh prior to use.

2) Reagent A: 135 mg FeCl₃·6H₂O was dissolved in 0.25 ml of water and diluted to 500 ml with glacial acetic acid containing 2% acetic anhydride.

3) Reagent B: 30N H₂SO₄.

4) Reagent C: Equal volumes of reagent A and B were mixed about one hour before use.

5) Standard Tryptophan: 5 mg of tryptophan was dissolved in 100 ml of water (50 μg/ml).

Procedure:

a) 100 mg of air-dried, powdered and defatted grain sample was taken into a small test tube.

b) 5 ml of papain solution was added to it, shaked well and the tube was closed.

c) The tubes were incubated at 65°C overnight.

d) The digests were cooled to room temperature, centrifuged and the clear supernatants were collected.

e) To 1 ml of supernatant, 4 ml of reagent C was added.

f) It was mixed in a vortex mixer and incubated at 65°C for 15 minutes.
g) The tubes were allowed to cool at room temperature and the orange-
red colour was read at 545 nm.

h) A blank set was run simultaneously with 5 ml papain alone and the
steps (c) to (g) were repeated again.

i) 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard tryptophan were pipetted out
and the volume was made to 1 ml with water. The colour was developed
following the steps (e) to (h).

*Calculation:*

The absorbance value of the blank was subtracted from that of the
sample and the tryptophan content was calculated from the standard
curve.

\[
\text{Tryptophan content in the grain sample} = \frac{\text{Tryptophan value from graph in } \mu\text{g} \times 0.096}{\text{Percent N in the sample}}
\]

\[= \frac{g}{16 \text{ g of N.}}\]

**3.5.6 SDS-PAGE of soluble proteins from seeds:**

The soluble proteins were electrophoresed on 12% SDS-PAGE
according to the method of Laemmli (1970).

**Solutions:**

**A) Acrylamide stock (30%)**

Acrylamide 30.0 g

Bisacrylamide 0.8 g

Volume was made up to 100 ml with water.

**B) 4X Tris-Cl/SDS, pH 6.8**

0.5 M Tris-Cl 6.05 g Tris.

0.4% SDS 0.4 g SDS

Dissolved in water, pH adjusted to 6.8 with HCl and volume was made
upto 100 ml.
C) 4X Tris-Cl/SDS, pH 8.8

1.5 M Tris-Cl 18.2 g
0.4% SDS 0.4 g

Dissolved in water, pH adjusted to 8.8 with HCl and volume was made upto 100 ml.

D) 10% Ammonium persulphate.

0.1 g ammonium persulphate was dissolved in 1 ml of distilled water. The solution was prepared fresh each time.

E) Sample buffer (4X)

Tris 1.52 g
Glycerol 20 ml
SDS 2.0 g
β-ME 2.0 ml
Bromophenol blue 1 mg

Dissolved in water, pH adjusted to 6.8 by HCl and volume was made upto 50 ml.

F) Electrophoresis buffer (5) pH 8.3:

Tris 15.1 g
Glycine 72.0 g
SDS 5.0 g

The contents were dissolved in distilled water and the volume was adjusted upto 1000 ml. For working standard, the solution was diluted with water in 1:4 ratio.

G) Staining solution:

50% Methanol
0.05% coomassie brilliant blue 250 R
10% Acetic acid
40% water
Coomassie brilliant blue 250 R was dissolved in methanol first and then acetic acid and water were added.

H) Destaining solution:

5% Methanol
7% Acetic acid
88% water

Separating gel preparation

<table>
<thead>
<tr>
<th>Components</th>
<th>For two 1.5 mm thick gels of 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>30 ml</td>
</tr>
<tr>
<td>Solution B</td>
<td>18.75 ml</td>
</tr>
<tr>
<td>Water</td>
<td>26 ml</td>
</tr>
<tr>
<td>Solution D</td>
<td>250 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Stacking gel preparation

<table>
<thead>
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<th>Components</th>
<th>For two 1.5 mm thick gels of 4.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Solution B</td>
<td>6.25 ml</td>
</tr>
<tr>
<td>Water</td>
<td>15.25 ml</td>
</tr>
<tr>
<td>Solution D</td>
<td>125 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Procedure

SDS-PAGE was carried out in vertical slab gel apparatus (Hoefer Scientific instrument). Separating gel was prepared first by separating gel solution into a vertical cassette leaving a margin of 3-4 cm on upper side. The gel surface was carefully overlaid with 1 cm of water to resist meniscus formation and the gel was allowed polymerizing at room temperature. After polymerization, the overlaid water was poured off
from the surface of the separating gel. Using filter paper, the remaining moisture was carefully absorbed without disturbing the gel surface. Then, stacking gel solution was poured into the mould after inserting the comb. The gel was allowed for polymerizing at room temperature. After the gel polymerization, the comb was removed and the wells were washed with water. The mould was attached to the assembly chamber and the whole unit was then immersed in electrode buffer. The apparatus was connected to the power supply (Pharmacia electrophoresis power supply EPS 500/400).

**Sample preparation and running the gel:**

Extraction of soluble protein was done by soaking the defatted powdered endosperm (1:4 w/v) overnight at 4°C in 50 mM Tris-Cl buffer (pH 7.6) containing 6 mM β-mercaptoethanol and subsequently grinding and homogenizing in a pestle and mortar. The cell paste suspension was centrifuged at 15,000 rpm for 10 min. at 4°C using REMI 2400 R centrifuge. 75 µl of the supernatant was mixed with 25µl sample buffer (Solution E) and boiled for 10 min. at 100°C. 80µl of samples were loaded into the wells and the electrophoresis was carried out under constant current of 50 mA at room temperature. The run was stopped when the dye front reached the bottom of the gel. The gel was then removed and stained overnight. Destaining of gel was carried out several times until the background became clear.

**Post-staining operation:**

After completion of process, the electrophoretic banding patterns were drawn according to scale and photographs taken. The relative fraction (Rf) value was calculated for each band, based upon the migration of the band, relative to the dye front.

\[
R_f \text{ value} = \frac{\text{Distance moved by band}}{\text{Distance moved by dye front}}
\]
3.5.7 ASSAY OF ENZYME ACTIVITY:

3.5.7.1 Peroxidase: (E.C.1.11.1.7)

The method as described by Sadasivam and Maniçkam (1996) was used for the isolation and assay of peroxidase activity.

One gram of freshly collected leaf sample was cut into small pieces and homogenized in 3 ml of 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 18000 g for 15 minutes at 4°C; the supernatant was used for assay of peroxidase activity.

The reaction mixture contained 3 ml phosphate buffer (0.1 M, pH 7.0), 0.05 ml of 20 mM guaiacol and 0.03 ml of 0.042% hydrogen peroxide ($\text{H}_2\text{O}_2$). The reaction was started by adding 0.1 ml of enzyme extract and mixed well with the reaction mixture. The absorbance was read at spectrophotometer at 435 nm and waited until the absorbance increased by 0.05. Immediately the time was recorded to calculate the required time in minutes ($\Delta t$) to increase the absorbance by 0.1.

Enzyme activity units per liter was calculated as: $3.18 \times 0.1 \times 1000 \over 6.39 \times 1 \times \Delta t \times 0.1$

Peroxidase activity in units per ml was then calculated and estimation done in triplicate.

3.5.7.2 Catalase activity (E.C.1.11.1.6.)

The method as described by Šadasivam and Manickam (1996) was used for the isolation and assay of catalase activity.

One gram of freshly collected leaf sample was cut into small pieces and homogenized in 10 ml of 0.0067 M phosphate buffer (pH 7.0) with a pre-cooled mortar and pestle. The homogenate was centrifuged at 18000G for 15 minutes. The sediment was stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking, and then repeated the extraction again. The supernatants were combined and used for the assay of catalase activity.
The reaction mixture contained 3 ml of H$_2$O$_2$-PO$_4$ buffer (0.16 ml of 10% w/v H$_2$O$_2$ diluted to 100 ml with 0.067 M phosphate buffer). The reaction was started by adding 0.01 ml of enzyme extract and mixed well with a glass rod. The absorbance was read in spectrophotometer at 240 nm. The time (Δt) in seconds required for a decrease in absorbance by 0.05 was recorded and this value was used for calculation of enzyme activity. The enzyme activity per ml of extract was calculated as:

\[
\text{Enzyme activity units/ml} = \frac{17 \times 10}{\Delta t \times 0.01}
\]

3.5.7.3 Acid phosphatase (E.C.3.1.3.2.):

The enzyme was isolated and assayed according to the method as described by Ikediobi et al. (1988).

Ten grams of fresh leaf sample was homogenized in 100 ml of 0.05 mM pre-chilled sodium acetate buffer (pH 5.0) containing 2% PVP with a pre-cooled mortar and pestle. The resulting homogenate was squeezed through four layers of muslin cloth into a pre-chilled beaker. The extract was centrifuged at 10000G for 20 minutes at 4°C and the resulting crude supernatant was used for assay of acid phosphatase activity.

The assay method was based on hydrolysis of p-nitro phenyl phosphate and the p-nitro phenol so released was estimated spectrophotometrically. The reaction mixture (2.1 ml) contained 2 ml of 10 mM p-nitro phenyl phosphate in 0.01 M sodium acetate buffer pH 5.0. The reaction was started by adding 0.1 ml of crude enzyme extract. After 10 minutes incubation at room temperature the reaction was terminated by adding 2 ml of 1 N NaOH. The absorbance of p-nitro phenol was measured at 400 nm against a reagent blank containing the substrate but without enzyme extract. The concentration of p-nitro phenol in micromole was calculated from its molar extinction coefficient of 18300 cm$^{-1}$M$^{-1}$. 
One unit of enzyme activity was defined as one micromole of p-nitro phenol formed per minute under the assay condition (Ikediobi et al. 1988).

3.5.7.4 Isozyme analysis

Isozyme analysis was carried out for three enzymes viz, peroxidase (E.C.1.11.1.7.), catalase (E.C.1.11.1.6.) and acid phosphatase (E.C.3.1.3.2.) and the banding patterns were studied in Polyacrylamide Gel Electrophoresis (PAGE) according to the method of Bloemendoal (1963).

Extraction of sample:

Extraction Buffer

Sucrose 20% solution was used for extraction of sample (Kay, 1996).

Extraction procedure

One gram of young, tender, fully expanded 30 days old leaves were homogenized with 5 ml of sucrose solution in a mortar and pestle. The crude extracts were then filtered through four layers of muslin cloth.

The filtrates were centrifuged at 8000 rpm for 10 minutes, using Himac CS80 rotor in a Hitachi CS120 Ultra centrifuge at 4°C. The clear supernatants were transferred to eppendrop tubes and mixed with 50% v/v glycerol in 2:1 ratio. The samples were then stored at -30°C for further use. All the above operations were carried out at 4°C.

Gel assembly preparation

Vertical polyacrylamide gel electrophoresis was carried out.

Preparation of stock solutions

Stock A. 0.3 M Tris-HCl (pH=8.9)

Tris = 3.633 g

Tris was dissolved in 50 ml distilled water, pH adjusted to 8.9 with 1.0 N HCl, and finally the volume was made to 100 ml by distilled water.
Stock B. 0.004 M Tris-HCl (pH=6.9)

Tris = 0.266 g

Tris was dissolved in 20 ml distilled water and pH was adjusted to 6.9 with 1.0 N HCl and finally the volume was made upto 50 ml by distilled water.

Stock C. 0.005 M Tris + 0.038 M glycine (pH=8.3)

Tris = 3.0275 g
Glycine = 14.263 g

Tris and glycine were dissolved in one liter distilled water and pH adjusted to 8.3 with 1.0 M Tris and finally the volume was made to 5 L by distilled water.

Stock D. 4.2 M acrylamide + 0.065 M N,N-methylenebisacrylamide.

Acrylamide = 29.854 g
N,N-methylenebisacrylamide (BIS) = 1.000 g

Acrylamide and BIS were added to 50 ml distilled water and finally the volume was made to 100 ml by distilled water.

Stock E. 1.4 M acrylamide + 0.16 M N,N-methylenebisacrylamide.

Acrylamide = 4.975 g
N,N-methylenebisacrylamide (BIS) = 1.233 g

Acrylamide and BIS were added to 25 ml distilled water and finally the volume was made to 50 ml by distilled water.

Stock F. 0.006 M Ammonium per sulfate

Ammonium per sulfate = 0.054 g
Distilled water (dH₂O) = 40 ml

(Stock F was prepared fresh just before gel preparation.)

Stock G. 10 mg/ml Riboflavin

Riboflavin = 50 mg
dH₂O = 5 ml
**Gel Mold Preparation**

The gel mold was prepared for the vertical zone electrophoresis apparatus (LKB Broma, 2001) system. The glass plates, spacers (1.5 mm thickness) and spacer mate were washed and they were assembled. The spacer mate and the two spacers were placed on a glass plate. The second glass plate was placed on top. Clamps were added and it was vertically set on flat surface. Spacer’s flush working glass was pressed and tightened with clamps and finally spacer mate was removed.

**Gel Preparation**

Gels were prepared by adding stacking gels

**Separating Gel Preparation**

8% separating gel was prepared with the following composition.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock D</td>
<td>17.04 ml</td>
</tr>
<tr>
<td>Stock A</td>
<td>8.0 ml</td>
</tr>
<tr>
<td>Stock F</td>
<td>32.0 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>32 μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>7 ml</td>
</tr>
<tr>
<td>Stock G</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

The chemicals were mixed and it was quickly dispersed into the gel mold with a Pasteur pipette. The top of the acrylamide solution was adjusted to approximately 20% below the top of the inside gel plate.

A thin layer of distilled water was dispersed on top of the acrylamide solution just after it began to polymerize to level the surface. The distilled water was removed after about 30 minutes.

**Stacking Gel Preparation:**

The stacking gel was prepared with the following composition
<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock B</td>
<td>17.04 ml</td>
</tr>
<tr>
<td>Stock E</td>
<td>8.0 ml</td>
</tr>
<tr>
<td>Stock F</td>
<td>32.0 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>32 µl</td>
</tr>
<tr>
<td>50% v/v glycerol</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Stock G</td>
<td>0.25 ml</td>
</tr>
</tbody>
</table>

The chemicals were mixed and it was quickly dispersed into the gel mold above the separating gel with Pasteur pipette.

The well form (comb) was inserted into the top layer of acrylamide solution before it began to polymerize.

The staking gel was polymerized within 30 minutes and the well form (comb) was removed carefully.

**Sample Loading**

50 µl of sample was loaded into each well immediately after polymerization of the gel with a micropipette. Upper and lower reservoirs were filled with stock C, making direct contact with both ends of the gel.

**Electrophoresis**

Gel was run at 250 volts and 50 mA current by a power supply system (Pharmacia Electrophoresis Power Supply EPS 500/400) for about 4 hours or until the tracking dye bromophenol blue migrated to the bottom of the gel.

**Staining of the gel**

After carefully removing the gels from the mold, slab gels were washed twice at 4°C with the buffer of the staining solution prior to staining. Staining was performed immediately for the identification of migration through the gels for 1-6 hours or until the bands appeared.
Composition of staining chemicals and gel incubation conditions are outlined below.

**Peroxidase** (Shaw and Prasad, 1978)

1. Peroxidase buffer
   - Sodium acetate = 5.74 g
   - Glacial acetic acid = 1.2 ml
   - dH₂O = 1 L
   - pH = 5.6
2. 0.1 M CaCl₂ solution = 4 ml
3. 3 amino-9-ethyl carbazole = 100 mg
   + N,N dimethyl formamide = 10 ml
4. 3% H₂O₂ = 2 ml

**Procedure:** Components were added in the buffer just before staining. Gels were incubated at room temperature.

**Catalase** (Scandalios, 1969)

1. Catalase buffer = 100 ml
   - Sodium phosphate, monobasic = 18.5 g
   - Sodium phosphate, dibasic = 17.9 g
   - Distilled water = 1000 ml
   - pH = 6.5
2. 2% KI solution = 100 ml
3. 0.03% H₂O₂ = 100 ml

**Procedure:** The gels were refrigerated in catalase buffer for 30 minutes followed by draining off the buffer and subsequently it was soaked in 2% KI solution. After 2 minutes, the KI solution was drained off and washed twice with tap water, H₂O₂ solution was added and band score was resolved.
Acid Phosphatase (Scandalios, 1961)

1. ACP acetate buffer = 160 ml
   Sodium acetate, trihydrate = 2.43 g
   Glacial acetic acid = 4.7 ml
   1.0 M MgCl₂ = 5.0 ml
   Distilled water = 1000 ml
   pH = 4.0
2. α-naphthyl acid phosphate = 200 mg
3. Fast Blue RR salt = 200 mg

Procedure: The stain components were allowed mixing well in stain buffer. Gels were incubated at room temperature.

Post staining operation:
After the development of bands, the stain was drained off and distilled water was added to the gels. The gels were placed in a light box and zymogram was drawn according to scale. Photograph of the plates was recorded. The relative mobility (Rm) value was calculated for each band based upon the migration of the band relative to the front.

3.5.7.5 Statistical analysis:
Level of polymorphism
The binary data generated by isozymes were used for determining polymorphism by dividing the polymorphic bands by the total number of bands. Polymorphism information content (PIC) was evaluated by using the formula given by Bhat (2002).

\[ \text{PIC} = 2 \pi_i (1 - \pi_i) \]

Where, \( \pi_i \) designates frequency of occurrence of polymorphic bands.

Data analysis
Data on isozymes were analyzed using the software package NTSYS-pc, Version 2.11 (Exter Software, NY, USA) (Rohlf, 2002).
Specific bands were scored as present (1) or absent (0) for each protein or isozyme sample and an index of genetic similarity or distance (1-F) calculated. F values were calculated by Jaccard’s similarity coefficient formula:

\[ F = \frac{a}{n - d} \]

Where, \( F \) = Similarity index

\( a = \) number of positive matches, \( i.e. \) the presence of band in both the samples

\( d = \) number of negative matches, \( i.e. \) the absence of band in both the samples

\( n = \) the total sample size including both the number of matches and unmatches

An agglomerative method of clustering genotypes was employed utilizing the unweighted pair group method with arithmetic averages (UPGMA) by using similarity matrix as input data (Sokal and Sneath, 1963). All the analyses were performed using software ‘NTSYS-pc, version 2.11X’ and ‘SPSS for MS Windows Release 7.5’.

**Measurement of genetic distance or similarity**

Since physio-morphological, biochemical and cooking quality traits were measured in different scales and hence the data were standardized with STAND module to have a mean of zero and a variance of one. Euclidean distance was computed by running SIMINT with standardized data. The average Euclidean distance is the dissimilarity coefficient. The larger the value of dissimilarity coefficient, greater the distance between the pair’s accessions. The formula for Euclidean distance (Sneath and Sokal, 1973) was as below.

\[ E_{ij} = \sqrt{\sum K (X_{ki} - X_{kj})^2} \]

For pair of genotypes i, j, only those values for K were used for which jKi and yKj were both present. This means that the different elements of the resulting matrix were based on different sample sizes.
Cluster analysis

The phenetic representation of genetic relationship among the varieties was revealed by Euclidean distance performed through cluster analysis using unweighted pair group method using arithmetic average (UPGMA). In UPGMA, the distance is based on the total number of taxa in the cluster. That is, if cluster 'i' contains Ti taxa, and cluster 'j' contains Tj taxa.

Then, $d_{ku} = \frac{(T_j \ d_{ki} + T_j \ d_{kj})}{(Ti + Tj)}$.

Similarly, matrices were used for cluster analysis in SAHN module. The SAHN module performs various clustering algorithms that Sneath and Sokal (1973) referred to as “Sequential, Agglomerative Hierarchical and Nested clustering methods”.

3.5.8 Determination of starch content: The starch from sugar free pellets was extracted in 52% perchloric acid at room temperature (Clegg 1956). The residue that remained after extraction of sugar was kept in an oven at 70°C until constant weight of the sample residue was attained and then transferred to a test tube. 5 ml of distilled water was added and kept in cool condition. 6.5 ml of 52% perchloric acid was added to it and stirred for 15 minutes in cool condition. Another 20 ml of distilled water was added and filtered in a 50 ml volumetric flask. To the residue again, 6.5 ml of 52% perchloric acid was added and stirred in cool condition for 15 minutes and filtered into the earlier 50 ml volumetric flask. The volume was adjusted to 50 ml by distilled water.

A suitable aliquot of the extract was taken and the volume was made to 2 ml with distilled water followed by 4 ml of anthrone reagent that was added in cool condition. The tubes were kept in boiling water bath for 8 minutes and cooled rapidly under running water. The absorbance of bluish green colour was read at 630nm. The glucose
content of the sample was determined by using standard graph. The value so obtained was multiplied by a factor 0.9 to determine the starch content.

3.5.9 Determination of amylose content: Starch is composed of two components namely amylose and amylopectin. Amylose is a linear or non-branched polymer of glucose. The glucose units are joined by $\alpha$-1-4 glucosidic linkages. Amylose exists in coiled form and Iodine is adsorbed within the helical coils to produce a blue colour complex. The blue colour complex was measured calorimetrically.

For determination of amylose content, 100mg of powder sample was taken in a test tube; 1ml of distilled ethanol was added to it. To the test tube, 10 ml of 1N NaOH was added and left over night. The volume was made upto 100 ml in a volumetric flask. From the final volume, 2.5 ml of the extract was taken in a 50 ml volumetric flask and 20 ml of distilled water was added and then three drops of 0.1% phenolphthalein were added. Subsequently, 0.1N HCl was poured to it drop by drop, till disappearance of pink colour to neutralize the base.

To this solution, 1 ml of iodine reagent was added and volume was made up to 50 ml and absorbance was read at 590nm. The iodine reagent was prepared by dissolving 1g iodine and 10g of potassium iodide (KI) in water and volume was made up to 500 ml.

Standard graph was prepared by standard amylose solution and colour development was done like that of sample solution.

Calculation:

Absorbance corresponds to 2.5 ml of the solution = $\chi$ mg. amylose.

\[ i.e. \ 100 \ ml \ contains \ = \ \frac{\chi \times 100 \ mg \ amylose}{2.5} \]

\[ = \ % \ amylose \]

3.5.10 Determination of amylopectin: The amount of amylopectin was obtained by subtracting the amylose content from that of starch.
3.5.11 Determination of total soluble sugar: Total soluble sugars were extracted by refluxing in 80% ethanol (Cerning and Guilbot 1973). Quantitative determination of total soluble sugar was carried out by colorimetric methods of Yemm and Willis (1954).

For this purpose, 100mg of the oven-dried sample was taken in a test tube to which few drops of ethanol and 2ml of distilled water was added. Test tube was stirred for 5 minutes with a glass rod; 5ml of boiling 80% ethanol was added and again stirred for 10 minutes. It was then filtered in a volumetric flask trying to keep the residue in the tube. The process was repeated for 4 to 5 times with 80% ethanol and total volume was made to 50ml by 80% ethanol. A suitable aliquot 0.2ml of the extract was taken and volume was made to 2ml with distilled water followed by 4ml of Anthrone reagent was added in cold condition. The tubes were then kept in boiling water bath for 8 minutes and then cooled rapidly and the absorbance of bluish green colour was read at 630nm.

**Calculation**

Absorbance corresponds to 0.2ml of test = Y mg of glucose.

\[ i.e. \quad 50 \text{ ml contains} = \frac{Y \times 100 \text{ mg}}{0.2} \quad \text{of glucose.} \]

\[ = \quad \% \text{ of reducing sugar.} \]

3.5.12 Determination of total reducing sugar: Total reducing sugar content was estimated by dinitro salicylic acid (DNS) method. DNS reagent was prepared by dissolving 1g of 3-5 dinitro-salicylic acid, 200mg crystalline phenol and 50mg sodium sulphate Na₂SO₄ in 100ml of 1% NaOH. In such an alkaline medium, DNS was reduced by glucose or any other reducing sugar to form 3amino-5nitro-salicylic acid, which was dark brown in colour and showed maximum absorbance at 500nm.

For the purpose of estimation of reducing sugar, 100mg of the sample was extracted with hot 80% ethanol twice (5ml each time). The
supernatant was collected and evaporated by keeping the tube on a water bath at 80°C. The residue was dissolved in 10ml of distilled water. 0.5ml of the extract was pipetted in test tube and volume adjusted to 3ml. Similarly different aliquots of working standard were taken in a series of test tubes and volumes were adjusted to 3ml, which acted as standard. To the each test tube, 3ml of DNS reagent was added and heated in boiling water bath for 5 minutes. In hot condition, 1ml of 40% Rochelle salt (Potassium sodium tartrate) solution was added. The tubes were cooled rapidly and the intensity of colour was read at 500nm.

The amount of reducing sugar present in the sample was calculated using the standard graph.

3.5.13 Determination of total non-reducing sugar: Total non-reducing sugar content was determined by calculating the difference between total soluble sugar and reducing sugar.

3.5.14 Determination of ash content: The ash content was determined as described in the AOAC (1970). For this, 5 g powdered sample was taken in a silica crucible, charred in low Bunsen flame and finally ignited at 600°C for 6 hours in the muffle furnace.

\[
\text{Ash content (g/100 g sample) } = \frac{\text{Weight of the ash}}{\text{Weight of the sample}} \times 100
\]

The estimations were done in triplicate and their mean was recorded as percentage of ash content in moisture free sample.

Preparation of mineral solution

The mineral solution was prepared according to the method described in AOAC (1970). For this purpose, ash was dissolved in HCl (1:1) on a water bath at 100°C and the solution was evaporated to dryness. After that, 4 ml hydrochloric acid and 2 ml glass distilled water were added, warmed and the acid soluble portion obtained after filtration made up to 100 ml in a volumetric flask with glass distilled water. This
solution was used for the estimation of minerals *viz.*, phosphorus, calcium and iron.

### 3.5.15 Determination of phosphorus content:

Phosphorus content was determined colorimetrically according to the method described by Fiske and Subbarow (1925).

To an aliquot (0.1 ml) of the mineral solution, 1 ml of ammonium-molybdate, 1 ml of hydroquinone, and 1 ml of sodium sulfite (Na$_2$SO$_3$) solution were added and mixed well after each addition. The volume was made up to 15 ml with water and the solution was mixed thoroughly. After 30 minutes, the optical density of the solution was measured in a spectrophotometer 106 against a reagent blank at 660 nm.

The amount of the phosphorus was calculated out from the standard curve of phosphorus. The estimations were done in triplicate and the mean value was recorded as gram of phosphorus per 100 g of moisture free sample.

### 3.5.16 Determination of calcium content:

Calcium was determined flame photo-metrically in a flame photometer, Systronics (Model – MK III) burner unit 121 at higher sensitivity.

The amount of calcium was calculated out from the standard curve prepared by using calcium solution of known strength. The estimations were done in triplicate and their mean value was recorded as mg of calcium per 100 g of moisture free sample.

### 3.5.17 Determination of iron content:

Iron content was determined colorimetrically according to the method described by Wrong (1928).

To an aliquot (6.5 ml) of the mineral solution, 1 ml of 30% sulphuric acid and 1 ml of saturated potassium persulphate solution were added and mixed thoroughly. After 20 minutes, the optical density of the
solution was measured in the spectrophotometer 106 at 540 nm against a reagent blank. The amount of iron was calculated out from the standard curve, which was prepared by using iron solution of known strength. The estimations were done in triplicate and their mean value was recorded as mg of iron per 100 g of moisture free sample.

3.6 Cooking quality traits

3.6.1 Alkali digestion value (Alkali spreading and clearing value)

The alkali digestion value was assayed as the extent of disintegration of dehusked rice treated with 1.7% KOH at 30°C for 23 hours (Little et al. 1958). The disintegration of grain was scored as low, low or intermediate, intermediate and high as per Table 3.2 mentioned below.

Table No: 3.2 Grades of alkali digestion of dehusked rice

<table>
<thead>
<tr>
<th>Numerical code</th>
<th>Description</th>
<th>Degree of alkali digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kernel not affected</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>Kernel swollen</td>
<td>Low</td>
</tr>
<tr>
<td>3</td>
<td>Kernel swollen, collar incomplete or narrow</td>
<td>Low/Intermediate</td>
</tr>
<tr>
<td>4</td>
<td>Kernel swollen, collar complete or wide</td>
<td>Intermediate</td>
</tr>
<tr>
<td>5</td>
<td>Kernel split or segmented, collar complete and wide</td>
<td>Intermediate</td>
</tr>
<tr>
<td>6</td>
<td>Kernel dispersed, merged with collar</td>
<td>High</td>
</tr>
<tr>
<td>7</td>
<td>Kernel completely dispersed</td>
<td>High</td>
</tr>
</tbody>
</table>

3.6.2 Gel consistency (mm): Gel consistency was analyzed based on the method described by Cagampang et al. (1973). The test classified the rice into three categories.
a) Very flaky rice with hard gel consistency (Length of gel less than 40 mm).
b) Flaky rice with medium gel consistency (Length of gel 40-60 mm).
c) Soft rice with soft gel consistency (Length of gel more than 60 mm).

3.6.3 Cooked rice kernel length (mm): The mean length of cooked rice kernel was measured in mm.

3.6.4 Cooked rice kernel lengthwise elongation ratio: The ratio of mean length of cooked rice kernel to mean length of dehusked rice was computed as lengthwise elongation ratio (Juliano and Perez 1984).

3.6.5 Cooked rice kernel breadth (mm): The mean breadth of cooked rice kernel was measured in mm.

3.6.6 Cooked rice kernel breadth wise elongation ratio: The ratio of mean breadth of cooked rice kernel to mean breadth of dehusked rice was computed as breadth elongation ratio.

3.6.7 Water absorption (%): It was the gram water absorbed after cooking per gram of uncooked rice expressed in percentage.

3.7 Statistical Analysis:

The following statistical and biometrical analyses were carried out based on the mean values of the observations recorded on sampled plants for different quantitative characters in this experimental study.

3.7.1 Analysis of variance
3.7.2 Genetic parameters
3.7.3 Analysis of covariance
3.7.4 Association studies
3.7.5 Path coefficient analysis
3.7.6 Classificatory analysis
3.7.7 Selection of genotypes
3.7.8 Analysis of variance for parents and crosses (F₁)
3.7.9 Heterosis study
3.7.10 Combining ability analysis

3.7.1. Analysis of variance:

For the analysis of variance of the mean data of each character, Fisher's method was followed. The total variation was partitioned into different components in the following way:

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Mean sum of square</th>
<th>Expected mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>((r-1))</td>
<td>MSr</td>
<td>(\sigma^2_e + g \sigma_r^2)</td>
</tr>
<tr>
<td>Genotypes</td>
<td>((g-1))</td>
<td>MSg</td>
<td>(\sigma^2_e + r \sigma_g^2)</td>
</tr>
<tr>
<td>Error</td>
<td>((r-1)(g-1))</td>
<td>MSe</td>
<td>(\sigma^2_e)</td>
</tr>
<tr>
<td>Total</td>
<td>((rg-1))</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genotypic variance was tested against the error variance by applying the F-test for significance as usual. Critical difference (C.D.) was calculated in order to test the difference of varietal mean as follows:

\[
C.D. = \text{S.E}_{d\pm} \times t \text{ for error degrees of freedom at 1\% or 5\% level of probability. S.E}_{d\pm} \text{ is the standard error of difference of varietal means to be tested.}
\]

\[
\text{S.E}_{d\pm} = \sqrt{\frac{2 \times \text{error MS}}{r}}
\]

Where, \(r\) = number of replications and

\(\text{MS} = \text{Mean sum of squares}\)

3.7.2 Genetic Parameters: The genetic parameters were calculated for various quantitative characters from the analysis of variance for that character.

3.7.2.1 Genotypic Variance \((\sigma^2_g)\): The genotypic variance was calculated according to Burton and Devane (1953).
3.7.2.2 Phenotypic Variance ($\sigma_p^2$): The phenotypic variance of each character was calculated by using the formula,

$$
\sigma_p^2 = \frac{\sigma_g^2 + \sigma_e^2}{r}
$$

Where, $\sigma_g^2 = \text{Mean sum of squares due to genotype}$

$\sigma_e^2 = \text{Mean sum of squares due to error}$

$r = \text{Number of replications}$

3.7.2.3 Genotypic Coefficient of Variation (GCV): Genotypic coefficient of variation is defined as the ratio of the square root of genotypic variance ($\sigma_g^2$) to the population mean ($\bar{X}$) and expressed in percentage. It was calculated by using the formula as suggested by Burton (1951, 1952) as follows.

$$
GCV = \frac{\sqrt{\sigma_g^2} \times 100}{\bar{X}}
$$

3.7.2.4 Phenotypic Coefficient of Variation (PCV): Phenotypic coefficient of variation is defined as the ratio of phenotypic variance ($\sigma_p^2$) to the population mean ($\bar{X}$) and expressed in percentage.

$$
PCV = \frac{\sqrt{\sigma_p^2} \times 100}{\bar{X}}
$$

3.7.2.5 Heritability ($h^2_{bs}$): Heritability in broad sense is the ratio of genotypic variance ($\sigma_g^2$) to the phenotypic variance ($\sigma_p^2$) and expressed as percentage. It was calculated by using the formula suggested by Hanson, Robinson and Comstock (1956) as follows.
3.7.2.6 Genetic Advance (GA): Genetic advance was calculated by using the formula as forwarded by Johnson, Robinson and Comstock (1955) as follows:

\[ G.A = k \cdot \sigma_p \cdot h_{bs} \]

Where, \( k \) = selection differential at 5% selection intensity.
\( \sigma_p \) = phenotypic standard deviation
\( h_{bs} \) = heritability in broad sense

3.7.2.7 Genetic advance in percentage of mean (GAPM): GA in percentage of mean was calculated by using the formula as,

\[ \text{GAPM} = \frac{G.A \times 100}{X} \]

Where, \( G.A \) = Genetic Advance
\( X \) = Population mean of the character

3.7.3 Analysis of Covariance (ANCOVA)

Analysis of covariance was carried out between yield and several yield attributing characters. Similarly, analysis of covariance was calculated between total soluble protein content with other biochemical characters and morphological characters by calculating the sum of product (SP) for replication, genotype and error respectively. Error sum of product was computed by subtracting the genotype sum of product and replication sum of product from total sum of product. Expected mean sum of products were calculated following the same principle as those for mean sum of squares. The outlines of the analysis of covariance are as follows:
3.7.4 Association Studies:

The genotypic, phenotypic and environmental correlation coefficients between yield per plant (Y) and several other yield attributing characters (X) under study were calculated by using the formula as suggested by Fisher (1954) and Al-Jibouri, Miller and Robinson (1958) as follows:

\[
\begin{align*}
\rho^g_{xy} &= \frac{\sigma^g_{xy}}{\sqrt{\sigma^g_x \cdot \sigma^g_y}} \\
\rho^p_{xy} &= \frac{\sigma^p_{xy}}{\sqrt{\sigma^p_x \cdot \sigma^p_y}} \\
\sigma^g_{xy} &= \text{Genotypic covariance between } x \text{ and } y. \\
&= \text{MSPg} - \text{MSPe} \\
\sigma^e_{xy} &= \text{Environmental covariance between } x \text{ and } y = \text{MSPe} \\
\sigma^p_{xy} &= \text{Phenotypic covariance between } x \text{ and } y = \sigma^g_{xy} + \sigma^e_{xy}
\end{align*}
\]

Where, \(\rho^g_{xy}\) = Genotypic correlation coefficient between the characters \(x\) & \(y\)

\(\rho^p_{xy}\) = Phenotypic correlation coefficient between the characters \(x\) & \(y\)

\(\sigma^g_{xy}\) = Genotypic covariance between \(x\) and \(y\).

\(\sigma^e_{xy}\) = Environmental covariance between \(x\) and \(y\) = MSPe

\(\sigma^p_{xy}\) = Phenotypic covariance between \(x\) and \(y\) = \(\sigma^g_{xy} + \sigma^e_{xy}\)

Where, \(\sigma^g_x\), \(\sigma^p_x\) and \(\sigma^e_x\) are the genotypic, phenotypic and environmental variance of \(x\) character.

Similarly, \(\sigma^g_y\), \(\sigma^p_y\) and \(\sigma^e_y\) are the genotypic, phenotypic and environmental variance of \(y\) character respectively.
The calculated genotypic, phenotypic and environmental correlation coefficients were tested for significance by “t” test as

\[ t = \frac{r \sqrt{n-2}}{\sqrt{1-r^2}} \] 

at \( n-2 \) degrees of freedom.

Where, \( r \) = coefficient of correlation and 
\( n \) = number of genotypes involved

**3.7.5 Path coefficient analysis:**

Path coefficients were calculated from the genotypic and phenotypic correlations according to the procedure given by Dewey and Lu (1959), which involves solving the simultaneous equations expressing the basic relationship between correlations and path coefficients. In general, it is determined from the equation:

\[ \sum_1^{(n-1)} r_{ij} \cdot p_{jn} = r_{iN} \]  

(1)

Where, \( N \) is the character taken as the effect and all other characters as possible causes, \( r \) and \( p \) are correlations and path coefficient respectively, \( i \) and \( j \) are column and row indices respectively and \( n \) is the total number of characters considered for analysis.

The path coefficients were obtained by solving a set of simultaneous equations of the formula:

\[ r_{ny} = P_{ny} + r_{n2} \cdot P_{2y} + r_{n3} \cdot P_{3y} + \ldots + r_{nn} \cdot P_{ny} \]  

(1)

Where,

- \( r_{ny} \) = represents the correlation between one component character and grain yield.
- \( P_{ny} \) = represent the path coefficients between the character and grain yield.
- \( r_{n2}, r_{n3}, ..., r_{nn} \) = represents the correlation between that character and each of the other yield component in turn.
In the matrix notation, the equation (i) may be written as:

\[
\begin{bmatrix}
T_{11N} & T_{12N} & T_{13N} & \cdots & T_{1(n-1)N} \\
T_{21N} & T_{22N} & T_{23N} & \cdots & T_{2(n-1)N} \\
T_{31N} & T_{32N} & T_{33N} & \cdots & T_{3(n-1)N} \\
& \cdots & \cdots & \cdots & \cdots \\
T_{(n-1)N} & T_{(n-1)1N} & T_{(n-2)2N} & T_{(n-3)3N} & \cdots & T_{(n-1)(n-2)N}
\end{bmatrix} = X 
\begin{bmatrix}
P_{11N} \\
P_{21N} \\
P_{31N} \\
\cdots \\
P_{(n-1)N}
\end{bmatrix}
\]

or \( r_{ij} = (r_{ij}) (p_{jN}) \)
\( \Rightarrow p_{jN} = (r_{ij})^{-1} / (r_{iN}) \)

To determine the values of inverse matrix \((r_{ij})^{-1}\) original square matrix was transferred in rows and column. The cofactors of the elements were then determined and divided by the determinant of the entire original matrix.

With the value of the inverse matrix, the \( (p_{jN}) \) was calculated. The indirect effects for a particular character through other characters were obtained by multiplication of direct path and particular correlation coefficient between those characters, respectively.

\[
\text{Indirect effect} = r_{ij} \times p_{iy}
\]

Where, \( r_{ij} = \text{correlation between the } i^{th} \text{ and } j^{th} \text{ characters.} \)
\( p_{iy} = \text{direct path of } i^{th} \text{ character on dependent character.} \)

The residual factor \((x)\) is given by:

\[
P_x^2 = 1 - \sum_{i=1}^{p} r_{iy} \cdot p_{iy},
\]

Where, \( p = \text{number of characters,} \)
3.7.6 Classificatory Analysis

Metroglyph analysis as proposed by Anderson (1957) was carried out in order to study the pattern of variation of sixteen characters of the genotypes with respect to the grain yield (ton/ha) and total soluble protein content (g/100g of oven dried sample). The sixteen characters are days to 50% flowering, plant height, effective branch tillers per hill, panicle length, grains per panicle, grain length, grain breadth, sterility percentage, 1000-grain weight, crude protein content, starch content, amylose content, amylopectin content, total soluble sugar content, total reducing sugar content and total non-reducing sugar content.

3.7.7 Selection of Genotypes: In order to select the promising genotypes from the experiment, ranking of genotypes was carried out on the basis of mean performance of seven physio-morphological characters like yield (tons/ha), panicle length, grains per panicle, sterility percentage, grain length, grain breadth and effective branch tillers per hill. Similarly another set of ranking of genotypes was done based on the mean performance of the genotypes with respect to eight different biochemical characters like total soluble protein content, crude protein content, starch content, amylose content, amylopectin content, total soluble sugar content, total reducing sugar content and total non-reducing sugar contents.

Thus, two sets of genotypes were selected based on: (1) yield and other yield attributing characters and (2) biochemical characters.

Rank correlation was worked out, prior to selection between yield and six other yield attributing characters; similarly for total soluble protein content and seven other biochemical characters using the rank correlation formula,
\[ r_{y_1} = 1 - \frac{6 \sum d_i^2}{n(n^2-1)} \]

Where, \( r_{y_1} \) = Correlation coefficient between grain yield and character 1,
\( d_i \) = Difference of ranks of "i"th variety between grain yield and character 1,
and \( n \) = Number of genotypes.

Rank correlation coefficients were tested by “t” test.

**Table No-3.3 A: Assigned ranks on the performance of genotypes with respect to seven physio-morphological characters**

<table>
<thead>
<tr>
<th>Assign rank</th>
<th>Yield (tons/ha)</th>
<th>Panicle length (cm)</th>
<th>Grains per panicle (nos)</th>
<th>Sterility percentage</th>
<th>Grain length (mm)</th>
<th>Grain breadth (mm)</th>
<th>Effective branch tillers/hill</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.91-5.48</td>
<td>30.0-28.8</td>
<td>310.0-286.5</td>
<td>0.90-2.50</td>
<td>10.50-10.07</td>
<td>2.50-2.43</td>
<td>20.0-18.3</td>
</tr>
<tr>
<td>2</td>
<td>5.48-5.05</td>
<td>28.8-27.6</td>
<td>286.5-263.0</td>
<td>2.50-4.10</td>
<td>10.07-9.64</td>
<td>2.43-2.36</td>
<td>18.3-16.6</td>
</tr>
<tr>
<td>3</td>
<td>5.05-4.62</td>
<td>27.6-26.4</td>
<td>263.0-239.5</td>
<td>4.10-5.70</td>
<td>9.64-9.21</td>
<td>2.36-2.29</td>
<td>16.6-14.9</td>
</tr>
<tr>
<td>4</td>
<td>4.62-4.19</td>
<td>26.4-25.2</td>
<td>239.5-216.0</td>
<td>5.70-7.30</td>
<td>9.21-8.78</td>
<td>2.29-2.22</td>
<td>14.9-13.2</td>
</tr>
<tr>
<td>5</td>
<td>4.19-3.76</td>
<td>25.2-24.0</td>
<td>216.0-192.5</td>
<td>7.30-8.90</td>
<td>8.78-8.35</td>
<td>2.22-2.15</td>
<td>13.2-11.5</td>
</tr>
<tr>
<td>6</td>
<td>3.76-3.33</td>
<td>24.0-22.8</td>
<td>192.5-169.0</td>
<td>8.90-10.50</td>
<td>8.35-7.92</td>
<td>2.15-2.08</td>
<td>11.5-9.80</td>
</tr>
<tr>
<td>7</td>
<td>3.33-2.90</td>
<td>22.8-21.6</td>
<td>169.0-145.5</td>
<td>10.50-12.10</td>
<td>7.92-7.49</td>
<td>2.08-2.01</td>
<td>9.8-8.10</td>
</tr>
<tr>
<td>8</td>
<td>2.90-2.47</td>
<td>21.6-20.4</td>
<td>145.5-122.0</td>
<td>12.10-13.70</td>
<td>7.49-7.06</td>
<td>2.01-1.94</td>
<td>8.1-6.40</td>
</tr>
<tr>
<td>9</td>
<td>2.47-2.04</td>
<td>20.4-19.2</td>
<td>122.0-98.5</td>
<td>13.70-15.30</td>
<td>7.06-6.33</td>
<td>1.94-1.87</td>
<td>6.4-4.70</td>
</tr>
<tr>
<td>10</td>
<td>2.04-1.61</td>
<td>19.2-18.0</td>
<td>98.5-75.0</td>
<td>15.30-16.90</td>
<td>6.33-6.20</td>
<td>1.87-1.80</td>
<td>4.7-3.00</td>
</tr>
</tbody>
</table>
### Table No-3.3 B: Assigned ranks on the performance of genotypes with respect to eight biochemical characters

<table>
<thead>
<tr>
<th>Assigned Rank</th>
<th>Crude protein content %N x 5.95</th>
<th>Soluble protein content (g/100g of oven dry sample)</th>
<th>Starch content (g/100g of oven dry sample)</th>
<th>Amylose content (g/100g of oven dry sample)</th>
<th>Total soluble sugar content (g/100g of oven dry sample)</th>
<th>Total non-reducing sugar content (g/100g of oven dry sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.20-12.58</td>
<td>6.72-79.88</td>
<td>25.62</td>
<td>84.83</td>
<td>1.20-0.367</td>
<td>0.960-0.887</td>
</tr>
<tr>
<td>2</td>
<td>12.58-11.96</td>
<td>6.45-78.45</td>
<td>24.52</td>
<td>83.73</td>
<td>1.11-0.340</td>
<td>0.887-0.814</td>
</tr>
<tr>
<td>3</td>
<td>11.96-11.34</td>
<td>6.18-77.02</td>
<td>23.42</td>
<td>82.63</td>
<td>1.02-0.313</td>
<td>0.814-0.741</td>
</tr>
<tr>
<td>4</td>
<td>11.34-10.72</td>
<td>5.91-75.59</td>
<td>22.32</td>
<td>81.53</td>
<td>0.93-0.286</td>
<td>0.741-0.668</td>
</tr>
<tr>
<td>5</td>
<td>10.72-10.10</td>
<td>5.64-74.16</td>
<td>21.22</td>
<td>80.43</td>
<td>0.84-0.259</td>
<td>0.668-0.595</td>
</tr>
<tr>
<td>6</td>
<td>10.10-9.48</td>
<td>5.37-72.73</td>
<td>20.12</td>
<td>79.33</td>
<td>0.75-0.232</td>
<td>0.595-0.522</td>
</tr>
<tr>
<td>7</td>
<td>9.48-8.86</td>
<td>5.10-71.30</td>
<td>19.02</td>
<td>78.23</td>
<td>0.66-0.205</td>
<td>0.522-0.449</td>
</tr>
<tr>
<td>8</td>
<td>8.86-8.24</td>
<td>4.83-69.87</td>
<td>17.92</td>
<td>77.13</td>
<td>0.57-0.178</td>
<td>0.449-0.376</td>
</tr>
<tr>
<td>9</td>
<td>8.24-7.62</td>
<td>4.56-68.44</td>
<td>16.82</td>
<td>76.03</td>
<td>0.48-0.151</td>
<td>0.376-0.303</td>
</tr>
<tr>
<td>10</td>
<td>7.62-7.00</td>
<td>4.29-67.01</td>
<td>15.72</td>
<td>74.93</td>
<td>0.39-0.124</td>
<td>0.303-0.230</td>
</tr>
</tbody>
</table>

3.7.8 Analysis of variance for parents and crosses (F1)

The mean values for all the quantitative characters were used for statistical analysis based on the following model.

\[ P_{ijk} = m + G_{ij} + b_k + e_{ijk} \]

Where,

- \( P_{ijk} \)= phenotype of the \( i^{th} \) genotype grown in \( k^{th} \) block
- \( m \)= general mean
- \( G_{ij} \)= effect of \( i^{th} \) genotype
- \( b_k \)= effect of \( k^{th} \) block
- \( e_{ijk} \)= random error associated with the \( i^{th} \) genotype in \( k^{th} \) block
The final analysis of variation was done as follows:

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom (df)</th>
<th>Mean sum of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>(r-1)</td>
<td>Mr</td>
</tr>
<tr>
<td>Genotypes</td>
<td>(g-1)</td>
<td>Mg</td>
</tr>
<tr>
<td>Parents</td>
<td>(p-1)</td>
<td>Mp</td>
</tr>
<tr>
<td>Crosses</td>
<td>(c-1)</td>
<td>Mc</td>
</tr>
<tr>
<td>Parents Vs Crosses</td>
<td>1</td>
<td>Mp Vs Mc</td>
</tr>
<tr>
<td>Error</td>
<td>(r-1)(g-1)</td>
<td>Me</td>
</tr>
</tbody>
</table>

Where,

- \( r \) = number of replications
- \( g \) = number of genotypes
- \( p \) = number of parents
- \( c \) = number of crosses
- \( Mr \) = replications mean sum of squares
- \( Mg \) = genotypes mean sum of squares
- \( Mp \) = parents mean sum of squares
- \( Mc \) = crosses mean sum of squares
- \( Mp \text{ Vs } Mc \) = parent Vs cross mean sum of squares
- \( Me \) = error mean sum of squares

The different variations were tested against error mean sum of squares by F-test for appropriate degrees of freedom.

To test the significance of difference between the genotypes, the following formula of critical difference (C.D.) was used.

\[
\text{C.D.} = \text{SE}_{dx} \times t \quad \text{at 5% or 1% level of significance for error degree of freedom.}
\]

Where, standard error of difference \( \text{SE}_{dx} = (2Me/r)^{0.5} \)

Standard error of mean \( \text{SE}_{mx} = (Me/r)^{0.5} \)
3.7.9 Estimation of heterosis

Heterosis was calculated over mid-parent (Briggle, 1963) and better parent (Hayes et al., 1965) by using the formula given below.

Heterosis over mid-parent (%) = \( \frac{F_1 - MP \times 100}{MP} \)  
(i.e. MP heterosis)

Heterosis over better parent (%) = \( \frac{F_1 - BP \times 100}{BP} \)  
(i.e. BP heterosis)

Where, \( F_1 \) = Mean of the particular \( F_1 \) individual  
\( MP \) = Mean of the two parents involved in a particular cross  
and  
\( BP \) = Mean of the better parent involved in the cross

Significance of heterosis was tested by 't' test as follows:-

\[ t_{mp} = \frac{F_1 - MP}{\text{S.E. ± of heterosis over mid-parent}} \]

\[ t_{bp} = \frac{F_1 - BP}{\text{S.E. ± of heterosis over better parent}} \]

Where, S.E. ± of heterosis over mid-parent = \( \sqrt{\frac{3Me}{2r}} \)

S.E. ± of heterosis over better parent = \( \sqrt{\frac{2Me}{r}} \)

3.7.10 Combining ability analysis:

Combining ability analysis was done following Griffing's (1956) method 2 (parents and \( F_1 \)'s excluding reciprocals), Model II.

3.7.10.1 Estimation of combining ability variance:

The sums of squares were calculated as follows:

\[ S_g = \frac{1}{P + 2} \left[ \sum (Y_i + Y_{ii})^2 - \frac{4}{P} \sum Y_i^2. \right] \]
\[ S_s = \sum \frac{1}{P+2} \sum (Y_i + Y_{ii})^2 + \frac{2}{(p+1)(p+2)} Y_{..}^2. \]

Where,

- \( S_g \) = sum of squares due to gca
- \( S_s \) = sum of squares due to sca
- \( p \) = number of parents
- \( Y_{ii} \) = mean value of the \( i^\text{th} \) parent
- \( Y_i \) = total of array of the \( i^\text{th} \) parent
- \( Y_{..} \) = grand total of \( \frac{1}{2} p(p-1) \) progenies and \( p \) parental values
- \( Y_{ij} \) = the progeny mean value in the diallel table

**Analysis showing the expectations of mean sum of squares is as follows:**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>Expected mean sum of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>gca</td>
<td>( (p-1) )</td>
<td>( S_g )</td>
<td>( M_g )</td>
<td>( \sigma^2e + \sigma^2s + (n+2) \sigma^2e )</td>
</tr>
<tr>
<td>sca</td>
<td>( p(p-1)/2 )</td>
<td>( S_s )</td>
<td>( M_s )</td>
<td>( \sigma^2e + \sigma^2s )</td>
</tr>
<tr>
<td>Error</td>
<td>( (p-1)(g-1) )</td>
<td>( S_e )</td>
<td>( M'_e )</td>
<td>( \sigma^2e ) = ( M'_e/r )</td>
</tr>
</tbody>
</table>

The test of significance:

- For gca variance: \( F \) with \( (p-1) \) and error d.f. = \( M_g/M_e \)
- For sca variance: \( F \) with \( p(p-1)/2 \) and error d.f. = \( M_s/M_e \)

**3.7.10.2 Estimation of gca and sca effects:**

General combining ability effect \( (g_i) \) of the \( i^\text{th} \) parent was calculated as:

\[ g_i = \frac{1}{(p+2)} \left[ \frac{1}{(Y_i + Y_{ii}) - \frac{2Y_{..}}{p}} \right] \]
Specific combining ability effect ($S_{ij}$) of the $ij^{th}$ cross was calculated as:

$$S_{ij} = Y_{ij} - \frac{1}{(p+2)} \left[ Y_i + Y_{ii} + Y_j + Y_{jj} \right] + \frac{2Y_{..}}{(p+1)(p+2)}$$

3.7.10.3 Standard error of the estimates:

Standard error of the gca and sca effects were computed as follows:

$$S.E.(g_{ij}) = \left[ (p-1) \sigma^2e/p(p+2) \right]^{1/2}$$

$$S.E.(s_{ij}) = \left[ 2(p-1) \sigma^2e/(p+1)(p+2) \right]^{1/2}$$

$$S.E.(d) \pm$$ for comparing two gca estimates:

$$S.E.(d)(g_i-g_j) = \left[ (2\sigma^2e/(p+2)) \right]^{1/2}$$

$$S.E.(d) \pm$$ for comparing two sca estimates:

$$S.E.(d)(s_{ij}-s_{kl}) = \left[ (2p\sigma^2e/(p+2)) \right]^{1/2}$$

Significance of the gca and sca effects were tested as follows:

$$t = \frac{g_i - 0}{SE(g_{ij})} \quad \text{and} \quad t = \frac{s_{ij} - 0}{SE(s_{ij})}$$

The ‘t’ values obtained were tested at error degree of freedom.

3.7.10.4 Parameters related to genetic components of variation:

Mean degree of dominance ($\bar{d}$): The mean degree of dominance was calculated following the ratio used by Verhalen et al. (1971)

$$\bar{d} = \left[ \frac{\sigma^2D}{\sigma^2A} \right]^{1/2}$$

Where, $\sigma^2D = \sigma^2\text{gca} = M_e-M_s$ and $\sigma^2A = 2\sigma^2\text{sca} = 2/p+2(M_e-M_s)$

Heritability in narrow sense ($h_{ns}^2$): It was calculated following the formula given below.

$$h_{ns}^2 = \frac{\sigma^2A}{\sigma^2p}$$

Where, $\sigma^2p = \text{Phenotypic variance}$