CHAPTER II

EXPERIMENTAL TECHNIQUES
CHAPTER II
EXPERIMENTAL TECHNIQUES

Seeds of the following six cultivars; three each of wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) exhibiting a wide range of plant height at maturity, were procured from Dr. G.S. Nanda, Senior Wheat Breeder; Dr. V.K. Saxena, Senior Maize Breeder of Punjab Agricultural University (PAU) Ludhiana (India); Dr. J.G. Bhowal, Senior Geneticist; Dr. Joginder Singh, Coordinator, All India Maize Improvement Project, of Indian Agricultural Research Institute (IARI), New Delhi (India) and Dr. V.G. Gawai, Director, Regional Fodder and Forage Research Station, Gandhinagar, Gujarat (India). During the course of this investigation following cultivars of wheat and maize were used.

<table>
<thead>
<tr>
<th>Name of the cultivar</th>
<th>Plant height at maturity (cm) under field conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHEAT</strong></td>
<td></td>
</tr>
<tr>
<td>A.1 WL-1562</td>
<td>80.0 (80.41 ±2.43)</td>
</tr>
<tr>
<td>A.2 PNC-1</td>
<td>105.0 (105.40 ±1.93)</td>
</tr>
<tr>
<td>A.3 C-306</td>
<td>125.0 (124.88 ±2.26)</td>
</tr>
<tr>
<td><strong>MAIZE</strong></td>
<td></td>
</tr>
<tr>
<td>B.1 J-202</td>
<td>137.0 (136.96 ±8.51)</td>
</tr>
<tr>
<td>B.2 Vijay</td>
<td>195.0 (194.60 ±4.45)</td>
</tr>
<tr>
<td>B.3 African</td>
<td>245.0 (244.73 ±4.045)</td>
</tr>
</tbody>
</table>

Note: Wheat cv.PNC-1 is a dwarf mutant of tall version cv. C-306 whereas maize cv.J-202 is a dwarf mutant of composite maize cv.Vijay

* Superscript letters denote the cultivar(s) number procured from that plant breeder.

** The values given in parentheses are the actual mean values noted under field conditions.
2.1 LABORATORY EXPERIMENTS

Seeds of average and uniform size of all the cultivars of wheat and maize, were surface sterilized with 0.1 % \( \text{HgCl}_2 \) for 10 min. They were then thoroughly washed with tap water, followed by several washings of distilled water. The washed seeds were placed over moistened filter paper (Whatman-1) in the petri dishes (15 cm). Petri dishes were incubated in dark at 25±2°C for wheat and 28±2°C for maize, in BOD incubator by covering them with several layered black cloth. After 24 hours, uniformly germinated seeds were transferred to other petri dishes (9 cm) containing 5 ml of nutrient solution as described by Arnon (1938) and Arnon and Hoagland (1940) and were transferred to light in a BOD incubator.

The nutrient medium was changed in all petri dishes everyday. Various analyses were done using 24, 48, 72, and 96 hours old seedlings after germination.

2.2 FIELD EXPERIMENTS

The experimental plots were ploughed and layered with enough farmyard manure. A basal dose of nitrogen fertilizer was provided at the rate of 50Kg per acre. Standard agronomic practices (viz. Package of Practices of Rabi and Kharif Crops (1981) P.A.U. Ludhiana) regarding sowing, irrigation and fertilization etc., were followed to optimise plant growth vis-a-vis yield. Sowing of wheat cultivars (viz., WL-1562, PNC-1 and C-306) was done in rows, 25 cm apart and at 4-5 cm depth, in the first week of November 1982, after pre-sowing
irrigation (rauni). Hoeing and thinning were done at 15 days after sowing and final distance of 5 cm was maintained between plant to plant. Sowing of maize cultivars (viz., J-202; Vijay; and African tall) was also done in rows, 75 cm apart and at 3-4 cm depth, in last week of June 1983, after presowing irrigation of the plots. Fifteen days after sowing, plants were thinned out at the time of first hoeing, keeping a plant to plant distance of 25 cm. Second hoeing was given at about 30 days after sowing.

2.3 ENZYME EXTRACTION AND PURIFICATION

2.3.1 Cytoplasmic Fraction:

The interactions between phenolic compounds and proteins in reference to the inhibition of enzyme activity have been reviewed by Looms and Battaile (1966). According to them phenolic compounds can bind to protein by hydrogen bonding or may be oxidised to quinones which copolymerise with protein through covalent bonding. Quinones may also condense to form tannins or brown pigments which inactivate enzymes and may cause precipitation of soluble proteins (Anderson and Rowan 1967). Numerous endogenous phenolic compounds are recognised as protein precipitants (Pirie, 1959; Stahman, 1963) and enzyme inhibitors (Hartdegen and Rupley, 1964; Goldstein and Swain, 1956; King 1971). Various additives to an extraction medium have been employed to remove these inhibitors. These include polyethylene glycol (PEG) (Clendemning, 1957; Badran and Jones, 1965), reducing agents (Anderson and Rowan, 1967; Stokes et al, 1968),
polyvinyl pyrolidone (PVP), both soluble and insoluble (Loomis and Battaile, 1966) and borate (King, 1971). In all these cases, a number of problems crop up, for example, it is not possible to remove PEG from proteins by gel filtration or dialysis. PVP (both soluble and insoluble) and borate have proved moderately successful in removing these inhibitors but they adversely affect some enzymes (King, 1971) and the dialysis of these substances is again a cumbersome process. Therefore, their use in every day work is most inconvenient. In the present work, chilled acetone for precipitation of proteins (Tang and Bonner, 1947, 1948; Smith and Stotz 1949; Galston and Baker, 1951) was found to be most suitable and therefore enzyme extracts were purified by chilled acetone precipitation procedure.

In case of seedling, root, shoots and endosperms were dissected out surgically at 24 hourly interval after germination. Field plants (at earing stage in wheat and tasselling stage in maize) were brought from field and their internodes were separated. The weighted amount of plant material was then homogenized in precooled mortar with help of pestle after adding a pinch of purified sand and 3 ml of cooled phosphate buffer (0.02 M, pH 6.4). Homogenate was then centrifuged at 10,000 g for 15 min., in a refrigerated centrifuge (REMI K-24) at -4 C. The supernatant served as a source for cytoplasmic fraction of enzyme. The residue was used for the extraction of wall bound fraction.
The supernatant was mixed with chilled absolute acetone (1:2) and incubated at 0-5°C for half an hour to precipitate the soluble proteins. It was again centrifuged at 3600 g for 10 min. The precipitated proteins were resuspended in 10 ml of phosphate buffer (0.02M, pH 6.4) and used as the cytoplasmic enzyme source for peroxidase, IAA oxidase, catecholase and amylase activities.

2.3.2 Wall Bound Fraction

The residue for wall bound fraction was washed several times with the extraction buffer till there was no cytoplasmic activity. Then 5 ml of 1M NaCl was added to the residue and kept at room temperature for 45 min., with frequent stirrings to release the ionically wall bound enzymes. The residue was washed twice and all the supernatants were mixed together. The final volume was made to 10 ml, which served as a source of ionically wall bound enzymes.

2.4 DISC ELECTROPHORESIS

The electrophoretic method of Ornstein and Davies (1962, 1964) and Hedrick and Smith (1968) using polyacrylamide gel (7.5% ; pH 8.9) for the detection of isoenzymes, was followed with some modifications: Disc electrophoresis requires a three phase system: an upper gel (spacer or stacking), in which the proteins concentrate initially; a lower gel (separation or running) in which the actual electrophoretic separation occurs and an electrode buffer.
The following recipe is for a normal analytical system:

1) 2.5% acrylamide upper gel pH 6.9
2) 7.5% acrylamide lower gel pH 8.9
3) Tris-glycine buffer pH 8.3

2.4.1 Preparation of Solutions

The solutions used for electrophoretic studies were of the following composition:

**STOCK-A**

1 N HCl ... ... ... 48.0 ml.
Tris (Tris hydroxy methyl amino methane) 36.3 g
TEMED (N N N N-tetra Methyl ethylene diamine) 0.23 ml.

pH 8.9
H O to make 100 ml.

**STOCK-B**

1 M H PO 3 4 ... ... ... 25.6 ml.
Tris ... ... ... 5.7 g
TEMED ... ... ... 0.46 ml.

pH 6.9
H O to make 100 ml.

**STOCK-C**

Acrylamide ... ... ... 30.0 g
Bisacrylamide ... ... ... 0.8 g
H O to make 100 ml.

**STOCK-D**

Acrylamide ... ... ... 10.0 g
Bisacrylamide ... ... ... 2.5 g
H O to make 100 ml.
2

**STOCK-E**

Riboflavin .......................... 4 mg

H O to make 100 ml.
2

**STOCK-F**

Sucrose .......................... 40 mg

H O to make 100 ml.
2

**STOCK-G** (catalyst)

Ammonium persulphate .......................... 0.14 g

H O to make 100 ml.
2
(made fresh each time)

**BUFFER-H**

Tris .......................... 3.09

Glycin .......................... 14.4 g

H O to make 100 ml.
2
(Used 20 ml per litre for final buffer, pH 8.3)

**TRACKING DYE-1 (.001 %)**

Bromophenol Blue .......................... 1 mg

H O to make 100 ml
2

**WORKING SOLUTIONS**

Separating Gel

2 parts .................................. Stock A
2 parts .................................. Stock C
1 part .................................. DW
8 parts .................................. Stock G
Spacer Gel

1 part .................................. Stock B
2 parts .................................. Stock D
1 part .................................. Stock E
4 parts ................................. DW

Stock solutions A and B required titration. Solutions were stored at 0-4°C. In stock C and D, acrylamide was dissolved first and then bisacrylamide was added since otherwise, it used to be difficult to get all the bisacrylamide into the solution.

The separating gels used to polymerize within 30-40 minutes after addition of catalyst, while spacer gels used to polymerize within 45 to 50 minutes after exposure to polymerizing light.

2.4.2 Preparation of Gels

Twelve tubes with open ends (0.5 cm inner diameter and 9.5 cm in length) were sealed at one end by wrapping the bottom of the tubes with plasteine tape. The separating gel (running gel) solution was gently pipetted into the tubes (1.7 ml each tube). Necessary care was taken to avoid air bubbles. A drop of water was softly layered at the top immediately to ensure flat miniscus of the gel. After approximately 35 to 40 min., gel polymerization was visualised as an appearance of sharp refractive boundary demarcating the separating gel from the water drop. This step was found to be critical in obtaining high resolution. After gel formation the water drop was removed first, with a flick.
of wrist and then with a piece of filter paper. In case of amylase, the separating gel was prepared by adding 1 part of 0.03% starch instead of 1 part of distilled water.

Again the gel tubes were mounted on the gel preparation rack and spacer gel solution was overlayered (0.3 ml) immediately followed by placing a drop of water. Spacer gel formation was accomplished by illumination of the gel mixture. Water drop was removed after photopolymerization of spacer gel.

2.4.3 Sample Preparation

Plant materials i.e., root, shoot and endosperm in case of seedling (Laboratory Experiments) and internodes of fully grown field plants (Field Experiments) were dissected and chilled. In preliminary experiments the amount of plant material necessary for obtaining moderate staining of gels was decided. The required amount of plant material (30 mg/ml approximately) was homogenized in precooled Tris-HCl buffer (0.01 M, pH 8.0; i.e. 30.3 mg Tris plus 1.34 ml of 0.1N HCl and final volume made to 100 ml with distilled water) in a glass mortar with help of pestle. The homogenate was centrifuged at 10,000g for 15 min in a refrigerated centrifuge (REMI K-24) at -4 C.

2.4.4 Electrophoretic Separation

The upper end of gel tubes was inserted into the silicon tube adaptor in the upper bath. The tubes were flushed with the adaptors. Air bubbles, if any trapped at the
lower end of the tubes were removed and the spaces were filled with electrolyte buffer with the help of syringe. Removal of air bubbles is absolutely essential for running of the sample through gel when the current is applied.

The enzyme (0.2 ml) was loaded over the spacer gel. This was followed by addition of 0.1 ml of sucrose (40%) to provide an extra density to enzyme for avoiding the problem of its mixing with the electrolyte buffer at the time of electrophoresis. Then, a drop of bromophenol blue (.001%) was added and this was followed by overlayering of electrolyte buffer (solution H) upto the top of the tubes to make a continuous liquid system without any air gap in between. The electrolyte buffer was poured both into upper bath (500 ml) and lower bath (500 ml).

The electrophoresis was carried out at low temperature (0 C). A constant current of 2 mA per tube was initially applied for 10 min., which was then raised to 4 mA per tube. Supply of current was stopped as soon as the bromophenol blue dye reached the lower end (2-5 mm above the bottom) of the tubes. The gel tubes were then removed from the bath and gels were extruded slowly by inserting a blunt point needle of the hypodermic syringe and applying slowly the pressurised flow of water. This was patiently performed with a precaution, not to make any scars on the gel during rimming. The gels were then stained for different isoenzymes.
2.4.5 Preparation of Zymogram

After staining of the gels, the bands were immediately traced on a graph paper. The intensity of each band was observed and expressed in arbitrary units. The distance travelled by each band and the tracking dye (bromophenol blue), into separating gel (from the interface with spacer gel) was measured. The results of electrophoretic separation have been presented schematically with mobilities calculated as relative distance traversed by the band with respect to distance travelled by bromophenol blue.

2.5 PROTEIN ESTIMATION

Protein contents in the cytoplasmic and wall bound enzyme extracts were determined using Folin reagent (Lowry et al., 1951) with slight modifications. 0.5 ml of enzyme extract (aliquot) was taken and to this, 5 ml of Lowry reagent C (4 ml of A, viz., 12.5% Na CO \(_2\) + 1 ml of B, viz., \(0.1\%\) CuSO\(_4\) ) was added. After incubation time of 10 min., 0.5 ml of Folin reagent was added and kept for 30 min., after mixing the contents thoroughly. Optical density (O.D) of the reaction mixture was recorded at 600 nm. Control was maintained by taking 0.5 ml distilled water (DW) instead of enzyme extract. The amount of enzyme protein was calculated and expressed as mg protein/gm fresh wt.

The specific methods of assaying enzymes, metabolites, and staining of isoenzymes etc. are presented in the concerned chapters.
2.6 STATISTICAL ANALYSIS

Correlation and regression analysis are used, to establish the relationship between different attributes/parameters of wheat and maize, confined to early stages of germination (Laboratory Experiments) as well as fully grown plants (Field Experiments). For the various sets of data, regression analysis has been carried out assuming linear, exponential and quadratic relationship between dependent \( Y \) and independent \( X \) variables. However, in each case only best fit regression equation (with highest \( R^2 \)) is selected and presented in the respective chapter(s).

For laboratory data, seedling growth and biochemical parameters are taken as dependent variables \( Y \) whereas plant height at maturity and growth periods are taken as independent variables \( X \).

For field data, biochemical parameters hitherto are treated as dependent variables \( Y \) whereas internode number and internode length of the fully grown plants are taken as independent variables \( X \). In all, following statistical equations are implied:

\[
\begin{align*}
y &= a + bx \\
y &= a + bx + cx \\
y &= a + bx + cx \\
y &= a + bx + cx \\
y &= a + bx + cx \\
y &= a + bx + cx
\end{align*}
\]

The computed values of correlation coefficient \( r \) have been
tested for their significance at 5% and 1% P levels (as per Sokol and Rohlf, 1969).

For harvest data (Chapter XI) 'Analysis of Variance' is employed for computation and significance of F-values at 5% and 1% P levels is tested and expressed therein.