Materials and Methods

Plant Materials:

Thirty *Dichanthium* accessions used for the present study represented collections from north central plateau (Bundelkhand region of Lalitpur and Chatterpur district of UP and MP) and southern plateau and hills (Dharwad and Bangalore, Karnataka) (Table 1). These accessions were collected from their natural habitats *i.e.*, grasslands and open fields and maintained in experimental fields of the Indian Grassland and Fodder Research Institute, Jhansi. The fresh and young plants from well grown and maintained tussock were transferred and grown in pots of uniform size (Figure 1). The tussocks were kept in uniform size by regular trimming. Fresh and young leaves from well grown tussocks of thirty *Dichanthium* genotypes in triplicate were studied to investigate their performance for various physiological and biochemical attributes under control and water stress conditions imposed by withholding the water. Molecular study was performed by isolating DNA from individual plants of each accession.

Morphological Observations:
Morphological data were recorded from well grown tussocks at 50% flowering stage.
Following observations were recorded and they were categorized as qualitative and quantitative traits.

Quantitative trait:

1. **Tussock height**: It was measured from base to top of the plant and recorded.
2. **Number of tillers per tussock**: Total number of tillers was counted and recorded.
Table 1: Agro-climatic regions and place of collection of 30 accessions of *Dichanthium annulatum* used in the present study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Accessions no.</th>
<th>Agro-climatic regions</th>
<th>Associated characteristics of regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IG97-234</td>
<td>North India central plateau</td>
<td>Arid to semi – arid, soils are alfisols and antisols, annual rainfall 800-1000 mm but restricted to the months of July, August and September and regions are characterized by very hot dry summers, maximum temperature touches 48°C</td>
</tr>
<tr>
<td>2</td>
<td>IG97-24</td>
<td>Lalitpur, north India</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IG95-30</td>
<td>Lalitpur, north India</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>IG97-192</td>
<td>North India central plateau</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>IG97-247</td>
<td>North India central plateau</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>IG97-241</td>
<td>North India central plateau</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>IG95-25</td>
<td>Lalitpur, north India</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>IG95-114</td>
<td>Lalitpur, north India</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>IG97-147</td>
<td>North India central plateau</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>IG97-170</td>
<td>North India central plateau</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>IG97-151</td>
<td>North India central plateau</td>
<td></td>
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<td>12</td>
<td>IG97-152</td>
<td>North India central plateau</td>
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<td>13</td>
<td>IG97-184</td>
<td>North India central plateau</td>
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<td>14</td>
<td>IG97-158</td>
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<td>15</td>
<td>IG97-118</td>
<td>North India central plateau</td>
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<td>16</td>
<td>IG97-233</td>
<td>North India central plateau</td>
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<td>17</td>
<td>IG97-189</td>
<td>North India central plateau</td>
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<td>18</td>
<td>IG97-132</td>
<td>North India central plateau</td>
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<td>19</td>
<td>IG97-218</td>
<td>North India central plateau</td>
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<td>20</td>
<td>IG97-130</td>
<td>North India central plateau</td>
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<tr>
<td>21</td>
<td>IG97-121</td>
<td>North India central plateau</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>IG97-244</td>
<td>Chatterpur, north India</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>IG97-245</td>
<td>Chatterpur, north India</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>IG97-144</td>
<td>Chatterpur, north India</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>IGKMFD-1</td>
<td>Dharwad, south India,</td>
<td>Humid, mixed red and gravelly soils, annual rainfall 700 – 1000 mm but distributed over a wide time period</td>
</tr>
<tr>
<td>26</td>
<td>IGTGD-4</td>
<td>Dharwad, south India</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>IGBANG-D-2</td>
<td>Bangalore, south India</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>IGKMD-10</td>
<td>Dharwad, south India</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>IG3108</td>
<td>Dharwad, south India</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>IGBANG-D-1</td>
<td>Bangalore, south India</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. *Dichanthium* accessions maintained in pots under net house conditions.
3. **Internodal length**: Length between two nodes were measured in centimeter.

4. **Longest leaf length and width**: It was measured in centimeters at the widest portion of leaf blade of the third leaf from the top of the main tiller. Average leaf width in millimeters from three leaves was calculated accordingly.

**Qualitative traits:**

- **Internodal color**: Color of nodes was categorized in three types as Red, light yellow and light green.
- **Nodal hairs**: Hairs on nodes were recorded as less, Medium and dense.
- **Awns**: Present or absent, if present then it was further divided into less, medium and dense.
- **Pits on floret**: Recorded as absent or present.
- **Culm thickness**: Culm thickness was measured by using vernier calipers in millimeter (mm).
- **Number of spikes and spikelets per plant at flowering stage**: Total number of spikes present in one plant was recorded.
- **Rachis hairiness**: Rachis was observed for presence or absence of hairs.
- **Type of stem**: It was determined by cutting stem transversely with sharp scalpel and recorded as solid or hollow.
- **Leaf hairiness**: Leaf surface was observed and recorded as either hairy or smooth.
- **Habitat**: Recorded as erect, semi erect and creeping.

**Measurement of leaf water relation parameters:**

Quantification of crop water status was made by measuring the leaf water relation parameters i.e., water potential (WP), osmotic potential (OP) and relative water content (RWC) at well irrigated condition (control) and under water stress (withholding of water for period of time).
Relative water content (RWC):
Relative water content (RWC) was determined by following the method of Barrs and Weatherley (1962). The expended and fresh leaves were cut into a uniform discs and weighed (Fresh weight). The leaves discs were than floated on distilled water for 6-8 hours at room temperature under the intense luminous light and determined the turgid weight by properly soaking leaves discs by tissue paper and weighed (Turgid weight), the same discs were oven dried at 80°C for 48 hour and dry weight was measured. RWC was calculated using the following equation.

\[
RWC = \frac{(FW-DW)}{(TW-DW)} \times 100
\]

Where FW is fresh weight, TW is turgid weight and DW is dry weight

Measurement of leaf water potential (WP):
Leaf water potential was measured at mid-photoperiod (10-12 noon) in fully expended leaves which were cut into uniform discs in three replicates from three plants of single genotype with thermocouple psychrometer and Wescor C-30 chambers connected to HR33T Dew point microvolt meter (Wescor Inc. USA). Water potential was recorded which is linear function of electro magnetic force produced by the temperature difference between the junction at the dew point temperature and the ambient temperature. The recorded value was later divided by proportionality constant (-0 75μ moles /bar) to get the value of water potential in bar which were further converted into Mega Pascal (MPa).

Measurement of osmolality and osmotic potential:
Osmolality was measured using leaf samples. Fresh and young leaves were collected and freezeed in sealed pack polythgin bags at -20°C. After 2 days the leaves were thawed at room temperature. The cell sap was extracted using compressed chamber (model LP-27, Wescor, USA) directly on the filter paper discs, and osmolality of cell sap was measured with a vapour pressure osmometer (5500, Wescor, Inc USA). Osmometer was calibrated with known concentrations (mmoles/kg) of NaCl solutions. Values obtained indicated the osmolality (mmoles/ kg).
These values were further converted into pressure units (MPa) using the following equation.

\[ \text{OP (MPa)} = -R \times T \times \text{moles/kg (osmolality)} \]

Where \( R \) is gas constant (0.008314) and \( T \) is the temperature (Kelvin Scale).

The obtained OP was further corrected for the dilution of symplastic sap by apoplastic water by using equation (OP+0.1 OP). Assuming 10\% apoplastic water (Kramer, 1983). The osmotic potential obtained at full turgor denoted as \( \text{OP}_{100} \) was calculated according to Wilson et al. (1979) using the following equation.

\[ \text{OP}_{100} = (\text{corrected OP} \times \text{RWC})/100 \]

**Measurement of osmotic adjustment (OA):**

Osmotic adjustment was calculated as the difference in osmotic potential at full turgor (\( \text{OP}_{100} \)) between stress and control treatments in each genotype.

**Estimation of proline:**

Estimation of low molecular weight free proline amino acid was determined in 500mg fully expanded leaves in 3\% sulphosalicylic acid in both control and stress plants by the method given by Bates et al. (1973). Five hundred milligrams leaves sample were homogenized in 10 ml of 3\% aqueous sulphosalicylic acid. The homogenates was filtered through Whatman No. 2 filter paper. Two ml of filtrate was taken in a test tube and 2.0 ml of glacial acetic acid and 2 ml of acid – ninhydrin (1.25g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid, with agitation until it is completely dissolved, stored at 4\°C and used within 24 hrs.) were added and mixed. The mixture was heated at 100 \°C for 1 hour in boiling water bath. After incubation reaction was terminated by placing the tube in ice bath. Four milliliter toluene was added to the reaction mixture and stirred well for 20-30 sec followed by extracting the solution using the separating funnel. Toluene containing proline was collected. Optical density (OD) was measured at 520 nm wave length. Standard plot of proline was made using pure proline solution of different concentration in place of tissues extract following the same procedure.
Estimation of \( \text{malondialdehyde (MDA)} \):

It was estimated in leaf tissues by following the procedure of Heath and Packer, 1968. Two hundred fifty milligram fresh leaf samples were homogenized in 5 ml cooled distilled water to fine slurry and homogenate was collected in 15 ml centrifuge tube. In it 5 ml TBA-TCA reagent (containing 0.5% thiobarbituric acid and 20% trichloroacetic acid) was added to the homogenate and mixed gently. After mixing the slurry was incubated in water bath at \( 95^\circ \text{C} \) for 30 min. The reaction was terminated by placing the tubes in ice bath for 10 minutes, and finally centrifuged at 10,000 rpm for 10 min. The supernatant was collected carefully and OD was recorded at 600 nm and 535 nm. Lipid peroxidation was determined by MDA content produced by thiobarbitric acid (TBA) reaction at low pH. The pink chromogen was measured at 535 and 600 nm for correction of the blank. The unspecific turbidity was corrected by substracting \( A_{600} \) from \( A_{535} \). Lipid peroxidation was expressed as milimoles per liter.

Estimation of total soluble proteins:

Proteins in ground leaf samples for enzymes, isozymes are estimated following the Lowry method (Lowry et al., 1951). In one ml of water 5 \( \mu \)l enzyme extract was taken and in the same test tube 5 ml of reagent C was added to each tube. After 10 min. of incubation 0.5 ml of Folin-coicalteau reagent was added and mixed well. All tubes were incubated at room temperature in dark for 30 min. In blank instead of enzyme extract water was taken. Blue color developed and OD was recorded at 660 nm. Standard graph of protein was constructed using known amount of bovine serum albumin (BSA) to calculate the amount of protein in unknown samples.

Cell membrane stability (Injury index):

Twenty five uniform leaf discs were taken in Pyrex tube containing 10 ml deionized distilled water and incubated at 25°C for 24 hr, then the electrical conductivity of the leachate was measured at 25°C directly using reading conductivity meter in both stressed and control (non stressed) tissues.
The tissue with leachate was then autoclaved, cooled to room temperature and total electrolyte was again measured by conductivity meter. The percentage membrane injury (I %) was calculated according to the formula of Blum and Ebercon (1981).

\[
I = 100 \times \frac{(1-T1/T2)}{(1-C1/C2)}
\]

Where C1 and C2 represents readings of electrical conductivity of control samples before and after autoclaving them respectively, and T1 and T2 represent readings of electrical conductivity of water stressed samples before and after autoclaving them respectively.

**Determination of Enzyme activity:**

**Preparation of enzyme extract:**
Fresh and young leaves were collected in icebox (4°C) for enzyme extraction. Plant samples were homogenized in three fold volume of cold extraction buffer (consisted of 50 mM Tris-HCl, pH 7.2, 10 % sucrose, 1.0 mM EDTA, 20mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM 2-mercaptoethanol (added freshly) and a pinch of polyvinyl pyrrolidone (PVP) in pre chilled pestle mortar to very fine slurry. The homogenate was centrifuged at 12,000 rpm for 20 min. The supernatant obtained was referred as enzyme extracts and used as enzyme source. It was kept in ice till the assay was carried out. An aliquot of the extract was used for protein determination (Lowry et al., 1951) and used to determine the specific activity of the enzymes by dividing the total obtained units in one gram fresh weight by total milligram protein in one gram fresh weight.

**Superoxide Di: nutase (SOD, E.C. 1.15.1.1)**

The SOD activity was determined by measuring its ability to inhibit photochemical reduction of Nitroblue tetrazolium (NBT) according to the method of Giannopoulos and Ries (1977) with suitable modifications. The 3.0 ml reaction
mixture contained 0.05M Na₂CO₃, 0.1mM EDTA, 63μM NBT, 13.0μM methionine, 20μl enzyme extract and 1.3μM riboflavin, riboflavin was added in last. The test tubes were placed under intense fluorescent light at sufficient distance at room temperature. After 20 minutes lights were switched off and OD was measured at 560 nm wavelength. The non irradiated samples were served as control. The reaction mixture lacking enzyme will develop maximum color due to maximum reduction of NBT. The reduction of NBT was inversely proportional to the enzyme activity. Thus to obtained ΔA, A₅₆₀ of particular set was deducted from A₅₆₀ of blank set (without enzyme). Percentage activity reduced was calculated and converted into units/min presuming.

50% inhibition = 1.0 unit

**Peroxidase (POD, E.C.1.11.1.17).**

Peroxidase activity was measured by the method of Chance and Machly (1955) where guaiacol was used as the substrate.

\[
\text{Guaiacol} + \text{H}_2\text{O}_2 \rightarrow \text{Oxidized guaiacol} + 2\text{H}_2\text{O}
\]

The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product was measured as peroxidase activity (POD) activity. In a 5 ml cuvette, 3 ml (0.1M) potassium phosphate buffer (pH-7.2) containing, 0.1ml (20 mM) guaiacol solution, 0.03 ml (12.3 mM) of hydrogen peroxide was taken. In it 0.05 ml of enzyme extract was added mixed and immediately placed the cuvette in sample chamber of the UV-VIS spectrophotometer against the reference which contains all the constituents except the enzyme extract. Increase in absorbance with time was recorded at A₄₃₆. The OD was measured at 15 seconds intervals for three min. This was done till the straight line appeared. Activity was determined as change in 0.01 OD per minute (one unit). Further it was divided by proteins content in mg present in extract to get specific activity unit per mg protein.
Pyroline 5-carboxylate synthetase (P5CS):

Pyroline 5-carboxylate synthetase (P5CS) activity was measured by following the method of Hayzer and Leisinger (1980). 0.1 ml supernatant was taken in a test tube followed by addition of 250 µl of assay mixture (containing 50 mM L-glutamate, 10 mM ATP, 20 mM MgCl₂, 100 mM hydroxylamine, 50 mM Tris buffer pH 7.0). The tube was incubated at 37 °C for 30 min. After incubation the reaction was terminated by adding 2.5% (w/v) FeCl₃ and 6% TCA in (2.5 M HCL). The protein precipitated was removed by centrifugation and absorbance of the supernatant was measured at 535 nm. The amount of γ-glutamyl hydroxamate produced was measured from the molar extinction coefficient of 250 l mol⁻¹ cm⁻¹ from the Fe³⁺- hydroxamate complex. All tubes were taken in triplicate and absorbance values were corrected for the values obtained at zero incubation time. The enzyme activity is represented in micromole min⁻¹ mg⁻¹ protein.

Polyacrylamide gel electrophoresis (PAGE):

Native polyacrylamide gel electrophoresis:

Discontinuous polyacrylamide gel electrophoresis was performed using a system based on Laemmli (1970).

Assembling the apparatus and preparation of gel solution:

Thoroughly cleared and dry glass plates along with proper spacers were fixed into gel casting unit (Banglore Genei, India) and tightly fixed with screw. The bottom of gel plates were sealed with 0.8% solution of molten agarose by gently pouring from sides of spacer with glass pipette and allowed the agar to solidify, resulting the sealing of bottom of glass plates. Two kinds of slab gels were casted based on their applications. They were either 1.5 mm or 1.00 mm thick and about 13 cm long and 14 cm wide when casted. Taking the account of size of the glass plates and thickness of spacer the amount (volume) of gel required was calculated. A 40 ml of resolving (separating) gel was prepared by mixing the various constituents. The required quantity of gel components were used for different percentage of gels preparation as given in table below.
Composition of resolving gels:

<table>
<thead>
<tr>
<th>Constituents</th>
<th>7% Gel</th>
<th>10% Gel</th>
<th>12% Gel</th>
<th>16% Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5M Tris-HCl buffer (pH 8.9)</td>
<td>10.0 ml</td>
<td>8.0 ml</td>
<td>10.0 ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Acrylamide solution (30%)</td>
<td>9.2 ml</td>
<td>13.3 ml</td>
<td>16.0 ml</td>
<td>21.3 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
<tr>
<td>Ammonium per sulphate (APS) 10%</td>
<td>200μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
<tr>
<td>*SDS (10%)</td>
<td>400.0μl</td>
<td>400.0μl</td>
<td>400.0μl</td>
<td>400.0μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>40.0 ml</td>
<td>40.0 ml</td>
<td>40.0 ml</td>
<td>40.0 ml</td>
</tr>
</tbody>
</table>

After mixing all the constituents, gel solution was carefully poured between the two glass plates, with leaving sufficient space for stacking gel. After pouring the gel it was over layered with fine layer of butanol. Gel was left for 30-45 min at room temperature for polymerization. After polymerization, over layered butanol was poured off and top of gel was washed with 3-4 times with deionized distilled water and drained completely all the fluids from top of the gel, remaining water was soaked with blotting paper. Stacking gel was prepared by mixing gel components along with desired concentration of acrylamide as given in table.

Composition of 4% and 5% stacking gel:

<table>
<thead>
<tr>
<th>Constituents</th>
<th>4%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (distilled and deionized)</td>
<td>6.01 ml</td>
<td>6.9 ml</td>
</tr>
<tr>
<td>1.014M Tris-Cl buffer (pH 6.8)</td>
<td>2.5 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>Acrylamide solution (30%)</td>
<td>1.33 ml</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>Ammonium per sulphate (APS) 10%</td>
<td>50.0μl</td>
<td>50.0μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10.0μl</td>
<td>10.0μl</td>
</tr>
<tr>
<td>*SDS (10%)</td>
<td>100.0μl</td>
<td>100.0μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>10.0 ml</td>
<td>10.0 ml</td>
</tr>
</tbody>
</table>

* Used when SDS gel electrophoresis was performed otherwise maintained the volume with distilled water.
Since the polymerization begins as soon as the TEMED added, immediately gel mixture was mixed gently and poured stacking gel solution directly on to the polymerized resolving gel, immediately after pouring cleaned teflon comb of defined well numbers and thickness was inserted into stacking gel solution making sure that no air bubble was trapped. Gel was kept in vertical position for 20 to 25 minutes at room temperature for polymerization. Comb was removed carefully form polymerized gel and wells were washed with distilled water and sealing agarose was removed. Gel plate was now installed in electrophoresis apparatus. Filled the anodal and cathodal reservoirs of apparatus with Tris-glycine electrode buffer. If any bubble that trapped at the bottom of the gel between glass plates was removed carefully.

Electrophoresis of gels:

Gels were electrophoresed at low temperature (4°C) to resolve isozyme. About 150 μg of protein sample mixed with 5 μl of bromophenol blue (tracking dye) was loaded into the well from cathodal end. The gel was run at 100 V till dye crossed the stacking gel and then at 200 V till the dye was ½-1 cm away from bottom.

Reagents:

1. Acrylamide and N, N'-methylene bis-acrylamide (30% stock solution):
Stock solution containing 29% (w/v) acrylamide and 1% (w/v) N, N'-Methylene bis-acrylamide prepared in deionized distilled H₂O and filtered before storing in dark brown beaker. Stock solution stored at 4°C.

For 100 ml volume:

\[
\begin{align*}
\text{Acrylamide} & = 29.0 \text{ g} \\
\text{Bis-acrylamide} & = 1.0 \text{ g}
\end{align*}
\]

Dissolve in 60 ml H₂O and finally made volume up to 100 ml.
2. Tris-buffer stock:
After the Tris base has been dissolved in distilled H₂O adjust the pH of solution using HCl to 8.9 and 6.8 for resolving and stacking Tris- buffer respectively.

3. N,N,N,N, - Tetramethylethylene diamine (TEMED):
Accelerates the polymerization of acrylamide and bis-acrylamide by catalyzing the free radicals from ammonium persulphate. Used directly as supplied.

4. Ammonium per sulphate (APS) stock solution (10%):
Stock solution was prepared by dissolving 1.0g Ammonium per sulphate in 10.0 ml distilled H₂O and stored at 4°C. Fresh solution was prepared weekly.

5. Tris-Glycine electrophoresis buffer (5X):
Stock solution (5X) was prepared by dissolving 3.0 g Tris base and 14.0 g of glycine in 900 ml distilled H₂O. In it 0.1% SDS added freshly in case of SDS PAGE.
2.8 g Glycine and 0.6 g Tris base and (1.0 g SDS)* was dissolved in 990 ml distilled water and final volume was made up to 1000ml to get working solution of running buffer.
* In case of SDS PAGE.

6. SDS stock solution (10% (w/v): 100 ml.
10 g SDS was dissolved in 60 ml D₂O and final volume was made up to 100 ml with distilled H₂O and stored at room temperature.

7. Composition of 1X SDS gel loading buffer:
   50 mM Tris-Cl pH 6.8
   100 mM 2- Mercapto ethanol
   2% SDS
   0.1% Bromophenol blue.
   10% Glycerol.

SDS-Polyacrylamide gel electrophoresis (PAGE):
SDS polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulphate (SDS) is an anionic detergent, which binds strongly to, and denatures proteins.
The number of SDS molecular bounds to a polypeptide chain is approximately half the number of amino acids residues in polypeptide chain. The protein-SDS complex carries net negative charge, hence move toward anode and the separation of protein is based on the size and molecular weight of protein.

The procedure of gel preparation and electrophoresis was same as discussed in native polyacrylamide gel electrophoresis, except that gel solutions i.e., resolving, stacking gel, sample loading butter and electrophoresis buffer contained 0.1% w/v SDS. Prior to loading the protein sample on the gel, protein samples were mixed with sample gel loading buffer to 1X, and boiled at 100°C for 3 min in boiling water bath followed by cooling the sample in ice. Immediately cooled samples were then loaded on to the wells.

Each well was loaded with approx 200 ng protein and gel was run initially for 10 min at 200 V and thereafter at 100 V until sample traveled through stacking gel and stack at the junction of blacking and resolving gel. Once stacking was completed gel was run at 200 V for 4-5 hrs. Till tracking dye reached to the bottom of gel once the run was completed gel plates were removed from electrophoresis apparatus and gels were separate from glass plated and kept for staining.

**Staining of native protein gel:**

Native protein gel was stained by immersing the gel in 100 ml staining solution containing methanol: distilled H₂O and acetic acid (4:5:1 ratio), 0.01% w/v brilliant blue R-250 (usually left for overnight). After staining gels were washed with distilled H₂O and immersed in destaining solution containing methanol: distilled H₂O and acetic acid (4:5:1 v/v) ratio for 1-2 hrs with occasional shaking.

**Staining of SDS-gel:**

Prior to staining SDS gel, it was immersed in 100 ml fixative solution (10% Trichloro acetic acid) for 1-2 hrs, followed by washing the gel with 2 to 3 times with distilled H₂O and gel was immersed in staining solution as discussed in
native protein gel staining followed by de-staining of in destaining solution. Destained gels were removed carefully and examined under light viewer.

**Agarose gel electrophoresis:**

Agarose is a linear polymer composed of alternating residues of D-and L-galactose joined by $\alpha-(1 \rightarrow 3)$ and $\beta (1 \rightarrow 4)$ glycosidic linkages. The chain of agarose form the helical fibres that aggregate into super-coiled structures with a radius of 20-30nm. Gelation of agarose results in a three dimensional mesh and channels whose diameters range from 50 nm to >200 nm

**Preparation of gel:**

Gel casting tray was cleaned and dried, adages of casting tray was sealed with gel sealing tape and set the casting tray on horizontal bench. Followed by comb of desired size and wells number was fixed into casting tray in such as manner that the comb was slightly above the surface of tray and fixed with comb stand.

Gel solution of agarose was prepared in electrophoresis buffer (0.5 x TBE) at appropriate concentration (Table) in screw cap bottle. Agarose was mixed in 0.5X TBE buffer and slurry was heated in microwave oven slowly with occasional swirling till all the grain of agarose were dissolved and gave clear transparent solution, transfer the bottle into a water bath at 65°C. When molten gel was cooled to 65°C. Ethidium bromide was added to final cone of 0.5 $\mu$g/ml (10 mg/ml) and mixed with gentle swirling. Luke warm agarose gel solution was carefully poured onto the gel casting tray and allowed the gel to set at room temperature. Gel took 30-45 minutes at room temperature to set completely. Once gel was set completely, comb and sealing tape was removed carefully. Casting tray was mounted into electrophoresis tank and filled the tank with 0.5 x TBE buffer till gel completely submerged into the buffer. DNA sample was mixed with 6X bromophenol loading dye to a final concentration of 1X and sample was loaded into the slots (wells) of the submerged gel using micropipette. Closed the lid of tank and electrical cords were connected so that the DNA would migrate
toward the anode (Red lead). Gel was allowed to run at 80V till bromophenol blue dye traveled the desired distance. After run, the gel was placed on gel documentation (Alpha Innotech, USA) system under UV light to examine. Photographs of ethidium bromide stained gel were taken.

**Protein extraction for enzyme activity assay:**
Fresh young leave from well grown tussocks were collected from both control and stressed plants and brought them to laboratory in ice bucket. Five hundred milligram leaves sample was ground in pre chilled pestal mortar in 1.5 ml of pre-chilled Tris-HCl (50 mM Tris-HCl, pH 7.5, 10 % sucrose, 1.0 mM EDTA and 1 mM 2-mercaptoethanol (added freshly) extraction buffer in presence of 20 mM PMSF and pinch of PVP powder. The sample was homogenized to fine slurry and centrifuge at 12000 rpm for 20 min at 4°C. The supernatant was collected and required quantity was used for isozyme and rest of extract was stored at 4°C.

**Polyacrylamide gel electrophoresis for isozyme:**
The analysis of four isozymes viz., peroxidase (POD, E.C. 1.11.1.17), esterase (EST, E.C. 3.1.1.2), polyphenol oxidase (PPO, E.C. 1.14.18.1) and superoxide dismutase (SOD, E.C. 1.15.1.1) was carried out using native polyacrylamide gel (10 %) electrophoresis method (Laemmli, 1970) with discontinuous buffer system. Gel was run at low temperature to resolve isozyme. About 150 μg of protein sample mixed with 5 μl of bromophenol blue (tracking dye) was loaded into the well from cathodal end. The gel was run at 100 V till dye crossed the stacking gel and then at 200 V till the dye was ½-1 cm away from bottom.

Gel was detached from the glass plates and placed in distilled water to avoid exposure to air and a piece of gel was cut at the right hand corner to mark the side. Gel was incubated in staining solution (substrate), the zones where the enzymes located in the gel were visualized due to the appearance of colored reaction product. Gels were photographed by using Olympus OM 2000 camera.
Staining of gels:

Peroxidase (POD, E.C. 1.11.1.17):
The gel was stained as described by Veech (1969). Gel was incubated in a solution containing 100 ml 0.05 M sodium acetate buffer (pH 5.6) containing 100 mg benzidine (dissolved by boiling) at room temperature. In the same buffer 10.0 ml 3% hydrogen peroxide was added gently mixed and 5 ml acetic acid was added to develop the color. The bright blue colored bands appeared. When the band was stained sufficiently, the reaction was arrested by immersing the gel in the large volume of 0.67% sodium hydroxide and 7% acetic acid solution for 10 min.

Esterase (EST, E.C. 3.1.1.2):
The gel was stained as described by Wendel and Weeden (1989). The gel was washed with 0.05M sodium phosphate buffer two to three times at 5-10 min intervals. Finally gel was immersed in 100 ml 0.05M sodium phosphate buffer (pH 6.0), and incubated at room temperature for 30 min. In an eppendorf tube 20 mg of alpha naphthyl acetate was dissolved in 1 ml of 60% acetone and in the same tube 50 mg fast blue RR salt was dissolved and poured onto already incubated gel. The gel was now incubated at 37°C for 20-30 minutes in dark with occasional shaking. Dark brown color bands appeared in the gel when desired intensity of band color was developed, the reaction was stopped by adding the mixture of methanol, acetic acid, water and ethyl alcohol in the ratio of 10:2:10:1. The stained gel was photographed using Olympus OM 2000 camera.

The gels was stained as described by Wendel and Weeden (1989). When the bromophenol blue dye touched the bottom of the gel it was removed and equilibrated in 100 ml 0.1M potassium phosphate buffer (pH 7.0) containing 0.1% p- phenylenediamine for 30 minutes at room temperature. When the incubation
was over, pyrocatechol was added to final concentration of 10mM in the same buffer. Dark brown bands appeared was photographed.

**Superoxide dismutase (SOD, E.C. 1.15.1.1):**

The gel was stained as described by Wendel and Weeden (1989). After the run was over gel was removed from the gel assembly and incubated in dark for 30 minutes in 100 ml of 0.05 M Tris-Cl buffer (pH 8.0) containing 2 mg riboflavin, 1 mg EDTA and 10 mg NBT. After the incubation gel was shifted to bright and intense light for 30 minutes and then gel was washed with distilled water. The bands appeared in form of negative bands against the blue background was photographed.

**Zymogram construction:**

The zymogram of gels was prepared by measuring the distance of each band from the point of separating gel and relative mobility \( R_m \) of each band was calculated as the ratio of distance traveled by the band to the tracking dye. Bands were numbered on the basis of increasing \( R_m \) values. Loci and alleles were subsequently numbered and lettered respectively. The isozyme patterns were defined by taking into account the numbers and positions of bands.

**Isolation of genomic DNA:**

Genomic DNA was isolated following N-Cetyl-N,N,N-trimethyl ammonium bromide (CTAB, method (Iqbal et al., 1997) with suitable modifications. Two grams of fresh and young leaves were ground in liquid nitrogen to fine powder and mixed with 4.0 ml CTAB total DNA extraction buffer (CTAB 2 % w/v, NaCl 1.4 %, Tris-HCl 100 mM pH 8.0, EDTA 20 mM and 2-Mercaptoethanol 100 mM, added freshly) and incubated at 65 °C for 1 hr with occasional swirling. Slurry containing nucleic acid was extracted with one volume of chloroform-isoamyl alcohol (24:1) then centrifuged at 8000 rpm for 15 min. Nucleic acid was precipitated by the addition of 0.6 volume of iso-propanol and kept at −20 °C for
2-3 hr. Nucleic acid pellet was dissolved in 10 mM TE buffer pH 8.0 and kept over night at 4°C. Dissolved nucleic acid was treated with 3μl/ml (5mg/ml stock) RNase by incubating them for 30 min at 37°C. After 30 min of incubation it was extracted twice with phenol/chloroform and finally with chloroform. DNA was precipitated using absolute ethanol and DNA pallet was finally washed with 70 % ethanol. Pallet was air dried and suspended in 1.0 ml TE buffer. Quantity and quality of DNA was quantified by UV spectrometry. OD was taken at A<sub>260</sub> and A<sub>280</sub> and A<sub>260</sub> / A<sub>280</sub> ratio was determined. Genomic DNA was visualized on 0.8 % agarose gels. Part of stock DNA was finally diluted with TE to final concentration of 5ng/μl for use in PCR.

**Agarose gel electrophoresis and restriction of genomic DNA:**

The DNA was electrophoresed on 0.8% agarose gel using 0.5X TBE buffer at 70 volts till loading dye was sufficiently traveled to visualize DNA in gel. After run the gel was stained in ethidium bromide. Along with the isolated DNA known amount of λ DNA digested with Hind III was loaded to check the size and amount of unknown DNA. The brief run was enough to reveal the quality and quantity of DNA. Five microgram (μg) of genomic DNA was restricted with Eco RI and Hind III in a final volume of 50 μl reaction mixture using 1X Eco RI and Hind III buffer respectively. The restriction of DNA was carried out at 37°C for 6-8 hrs. The restricted DNA was electrophoresed using 0.7% agarose gel containing ethidium bromide. The Hind III digest λ DNA was used as molecular weight marker. The gel was run at 70 volt using 0.5X TBE electrode buffer. When run was over the gel was photographed using Polaroid and SLR camera.

**Polymerase chain reaction PCR:**

**Random Amplified polymorphic DNA (RAPD):**

RAPD-PCR reactions was performed in 20 μl of reaction mixture containing 67 mM Tris-HCl (pH 8.0), 16.6 mM (NH₄)₂SO₄, 0.45 % v/v BSA, 3.5 mM MgCl₂, 150 μM of each dATP, dTTP, dCTP, dGTP, 7.5 pmoles (15ng) primer, 0.5 unit
Taq polymerase and 25 ng genomic template DNA. PCR product was visualized with ethidium bromide after electrophoresis on 1.6 % agarose gel.

**Composition of reaction mixture for single RAPD Reaction (25μl):**

10X PCR buffer\(^{SM}\) - 2.0μl
MgCl\(_2\) (25mM) - 2.8μl
dNTPs mix (10mM each) - 1.25μl
Primer (10mM) - 1.2μl
Taq DNA Pol(3U/μl) - 0.15μl (0.5unit)
H\(_2\)O - 10.5μl
Genomic DNA - 5.0 μl (Diluted DNA to 5ng/μl to get 25ng)

Mixed all the components (except genomic DNA) and 15μl of the mixture was added in each single reaction.

The reaction was performed in thermal cycler (PTC 200, MJ Research, USA) with cycling program

Cycle- 1

94°C - 1.0 min
37°C - 1.0 min
72°C - 2.0 min

Repeat above steps in cycle-1 for 40 times

Cycle-41st

72 °C for 10 min (Extension)

and finally 4 °C for ever. Amplified products were stored at 4 °C.

**Agarose gel electrophoresis for RAPD-PCR product:** PCR product was separated on a 1.6% agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2μl 5xDNA
loading dye and total samples were loaded on the gel. Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was run at 70V for four hours. The amplified product was visualized under UV trans-illuminator and was photographed using Polaroid and SLR camera.

**STS PCR Procedure:**

The PCR amplification of STS loci was conducted following the procedure given by Liu *et al.*, (1996) with certain essential modifications like adjustment of genomic DNA concentration and inclusion of primer extension step for 5 min as a last step of amplification in thermal cycler.

STS-PCR reaction was performed in 25μl volume reaction mixture containing 78.2 mM Tris-HCl pH (8.0), 19.4 mM (NH₄)₂SO₄, 233μg/ml BSA, 0.53% TritonX-100 5.8 mM MgCl₂, 130 μM of each dATP, dTTP, dCTP, dGTP, primer, 1.0 unit Taq polymerase and 25 ng genomic template DNA.

**Composition of reaction mixture for single STS-PCR:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer&lt;sup&gt;(SM)&lt;/sup&gt;</td>
<td>3.3μl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>6.6μl</td>
</tr>
<tr>
<td>dNTPs mix (10mM each)</td>
<td>2.9μl</td>
</tr>
<tr>
<td>Primer (10mM)</td>
<td>2.0μl</td>
</tr>
<tr>
<td><em>Taq</em> DNA Pol(3U/μl)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>10.5μl</td>
</tr>
<tr>
<td>Genomic DNA (Diluted DNA to 5ng/μl to get 25 ng)</td>
<td>5.0μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>25μl</td>
</tr>
</tbody>
</table>

Mixed all the components (except genomic DNA) and 20μl of the mixture was added in each single reaction.
PCR conditions for STS-PCR. Following programme was used in PCR amplification for STS loci.

94°C - 60 second
55°C - 60 second
72°C - 90 second

Repeat above steps for 32 cycles

One cycle at 72°C for 5 min and finally at 4°C for ever. Amplified product was stored at 4°C.

**Agarose gel electrophoresis for STS-PCR product:**

PCR products were separated on a 1.6% agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2ul (5x) DNA loading dye and were loaded on the gel. Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was ran at 80 volts for four hours. The amplified product were visualized under UV trans-illuminator and were photographed were taken using gel documentation system.

**ISSR-PCR Procedure:**

ISSR-PCR reaction was performed in 25ul volume reaction mixture containing 67.0 mM Tris-HCl pH (8.0), 16.6 mM (NH₄)₂SO₄, 4.0μg BSA, 0.396% TritonX-100 2.0 mM MgCl₂, 150 μM of each dATP, dTTP, dCTP, dGTP, 0.4μM primer, 1.0 unit Taq polymerase and 25 ng genomic template DNA.

**Composition of reaction mixture for single ISSR-PCR**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer (SM)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>dNTPs mix (10mM)</td>
<td>1.5 μl</td>
</tr>
</tbody>
</table>
Primer (10mM)  -  2.0 μl

*Taq DNA Pol(3U/μl)  -  0.3 μl (1.0 unit)

H2O  -  11.7 μl

Genomic DNA  -  5.0 μl  (Diluted tamplet DNA to 5ng/μl to get 25 ng)

Final volume  -  25.0 μl

Mixed all the components (except genomic DNA) and 20ul of the mixture was added in each single reaction.

Following programme was used to amplify ISSR markers.

**Cycle -1**

94°C  -  2.0 min.

**Cycle- 2**

94°C  -  2.0 second

50°C  -  1.0 min.

72°C  -  1.30 min

Repeat above steps in cycle-2 for 34 times

**Cycle-35**

72°C for 10 min

And finally at 4°C for ever. Amplified product was stored at 4°C

Agarose gel electrophoresis for ISSR-PCR product: PCR product was separated on a 1.6 % agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2ul 5X DNA loading dye, and were loaded on the gel. Along with the unknown samples 20 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was run at 80 volts for five hours. The amplified product were visualized under
Data analysis and development of dendrograms:

For isozyme markers the parameters estimated for genetic variation were percentage of polymorphic loci (Pp), mean number of alleles per polymorphic locus (Ap), mean expected heterozygosity based on unbiased estimate of Nei (1978) and allele frequency. Allele frequencies were used to estimate expected heterozygosity. A locus was considered polymorphic when more than one allele was found in an accession. The proportion of polymorphic loci was calculated by dividing the number of loci polymorphic by the total number of loci analyzed. The mean number of alleles per polymorphic locus was determined by summing all the alleles observed at polymorphic loci and dividing this sum by the number of polymorphic loci (Hamrick and Godt, 1997). Genetic diversity ($H_e = \text{Hardy-Weinberg expected heterozygosity}$) (Weir, 1989) was calculated for each locus (including monomorphic and polymorphic loci) by:

$$H_e = 1 - \sum x_i^2$$

Where $x_i$ is the mean frequency of the $i^{th}$ allele pooled across the accessions.

The binary data generated on the basis of presence (1) and absence (0) of the bands in isozyme, RAPD, STS, and ISSR, was analyzed for genetic similarity among the accessions based on Dice's similarity coefficients ($s$), which was also converted to distance measures ($d$) using the formula $d = 1-s$. Dendrogram was constructed by Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using the Un-weighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. A RAPD band was considered polymorphic if the band was present in some accessions and absent in others, monomorphic if the band was present among all the accessions.
Polymorphic information content (PIC) value was calculated of individual primer to evaluate discriminatory power of markers. The PIC value was calculated applying the formula of Roldan-Ruiz et al. (2000): \( \text{PIC}_i = 2f_i(1-f_i) \), where \( f_i \) is the frequency of the amplified allele (band present) and \( (1-f_i) \) is the frequency of the null allele (band absent) of marker \( i \). Marker index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell et al., 1996).

**Boot strap analysis:-**

Boot strap analysis further authenticated the genetic relationship among the accessions. The reliability of dendrogram was evaluated with 1000 Boot straps using Win Boot software (Yap and Nelson, 1996).

**Correlation of biochemical data with DNA patterns:**

**Significance of the value:**

Standard excel software was used to calculate the degree of freedom, LSD, mean of square, t-test single and two way ANOVA.

**Association of RAPD, ISSR and STS DNA finger prints with stress responsive traits.**

Regression analysis was conducted using the data of stress responsive traits and data of three molecular marker systems viz., RAPD, STS and ISSR. In total eight stress parameters were used for association in all 30 *Dichanthium* accessions. These parameters were osmotic adjustment (OA), osmolality, MDA content, proline content, injury index, peroxidase activity, superoxide activity, and P5CS activity. Association of RAPD, STS and ISSR markers with these eight stress responsive traits were investigated using multiple regression analysis. The amount of change in the traits were treated as dependent variables and various RAPD, STS and ISSR bands (scores as 1 for presence of band and 0 for absence) were treated as independent variables. The regression analysis was based on the model:
Where $Y$ is the trait (parameter), $m$ the RAPD, STS and ISSR markers, $b$ partial regression coefficient, $d$ the between accession residue which is left after regression.

Solutions and buffers for plant DNA isolation:
1) 0.5M EDTA (pH 8.0): Dissolved 37.22g EDTA di-sodium salt and 4.0g Sodium hydroxide in 150 ml H$_2$O and adjusted the pH by NaOH solution, final volume was made up to 200 ml.

2) 1M Tris-Cl pH (8.0): Dissolved 24.23g Tris base in 150 ml distilled water Adjust to the pH 8.0 by HCl and made up final volume to 200 ml with water.

3) 5M NaCl: Dissolved 58.44g NaCl in distilled water and made up final volume to 200 ml with water.

4) CTAB (10%) W/V: Dissolved 200g CTAB in 180 ml distilled water. CTAB was dissolved by warming the solution. The final volume was made up to 200 ml.

5) 10mM TE Buffer (pH 8.0): 2ml Tris-HCl from 1M Tris–HCl stock and 0.4 ml EDTA from 0.5M EDTA stock. The final volume was made up to 200 ml with D. water.

6) 5x Tris Borate EDTA Buffer or TBE Buffer (pH 8.0): Dissolved 27g Tris base, 13.75g boric acid, 10ml (0.5M) EDTA in 400ml distilled water and made to final volume to 500 ml with water.

7) DNA Lysis Buffer(CTAB):

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM EDTA</td>
<td>8ml (0.5M EDTA)</td>
</tr>
<tr>
<td>100mM Tris- HCL</td>
<td>20ml (1M Tris-Cl pH8.0)</td>
</tr>
<tr>
<td>1.4M NaCl</td>
<td>56ml (5M NaCl)</td>
</tr>
<tr>
<td>2% W/V CTAB</td>
<td>40ml (10% CTAB)</td>
</tr>
<tr>
<td>100mM 2-ME Added freshly</td>
<td></td>
</tr>
</tbody>
</table>

Mixed and made up final volume to 200 ml with D. water.

8) DNA loading dye (Bromophenol blue): 50.0ml
Final concentration Stock concentration
0.1M EDTA 10ml (0.5M)
40% Sucrose 20g
25% Bromophenol blue 125mg
Final volume was made up to 50 ml with distilled water.

9) 10X Taq buffer:

<table>
<thead>
<tr>
<th>Stock concentration</th>
<th>Vol./amt. taken</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-Cl (pH 6.8)</td>
<td>6.7 ml</td>
<td>0.67M</td>
</tr>
<tr>
<td>1M Ammonium sulphate</td>
<td>1.66 ml</td>
<td>0.166M</td>
</tr>
<tr>
<td>100% Triton X-100</td>
<td>0.45ml</td>
<td>4.5% (V/V)</td>
</tr>
<tr>
<td>BSA</td>
<td>20mg</td>
<td>0.2%</td>
</tr>
</tbody>
</table>
Final volume was made to 10 ml with HPLC grade sterile water.

**Solution and buffers for PAGE:**

1) Resolving gel buffer pH 8.9: Dissolved 18.15 gm Tris base in 60 ml distilled water. Adjusted the pH 8.9 by adding HCL and made the final volume 100 ml with water.

3) Stacking gel buffer: (0.6M Tris base pH 6.7) Dissolved 6.1g Tris base in 60 ml distilled water and adjusted pH 6.7 by adding HCL and made up final volume 100 ml.

4) 10% Ammonium per sulphate solution (APS): Dissolved 100 mg APS in 1ml distilled water.

5) Running electrode buffer (pH 8.3):

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>0.6g</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2.8g</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1 liter</td>
<td></td>
</tr>
</tbody>
</table>

6) Staining solution:
Coomassie brilliant blue-R-250- 100 mg
Methanol - 40 ml
Acetic acid - 10 ml
Made up final volume upto 100 ml with D.water.

7. De-staining solution:
Methanol - 40 ml
Acetic Acid - 10 ml
Distilled Water - 50 ml

**Extraction buffers for isozyme and PAGE analyses:**
0.05M Tris Base - 0.625g
Sucrose (5% w/v) - 5.0g
EDTA - 168 mg
2-ME - 100μl

Dissolved in 60 ml distilled water and adjusted the pH 7.5 by adding 1.0 N HCl and made up the final volume to 100 ml.

**Reagent C (Alkaline copper solution):** Mixed 50 ml of reagent ‘A’ and 1 ml of reagent ‘B’ prior to use.

(a) **Reagent A:** 2% Sodium carbonate in 0.1N Sodium hydroxide.
(b) **Reagent B:** 0.5% Copper sulphate (CuSO₄. 5H₂O) in 1% Potassium sodium tartrate.

Alkaline copper tartrate:
(a) Dissolved 2.5 g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20g anhydrous sodium sulphate in 80 ml water and made up final volume to 100 ml.
(b) Dissolved 50 g copper sulphate in a small volume of distilled water. Added one drop of sulphuric acid and made up final volume to 100 ml.
Mixed 4 ml of B and 96 ml of solution A before use.
PMSF (1M) stock: Dissolved 17.4 mg PMSF in 1.0 ml ethanol and stored in freeze.

2X Sample buffer for SDS PAGE:

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>volume taken</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125M Tris HCl</td>
<td>0.125 ml</td>
<td>1M</td>
</tr>
<tr>
<td>Water</td>
<td>0.175 ml</td>
<td></td>
</tr>
<tr>
<td>4% SDS</td>
<td>0.4 ml</td>
<td>10%</td>
</tr>
<tr>
<td>20% Glycerol</td>
<td>0.2 ml</td>
<td>100%</td>
</tr>
<tr>
<td>10% 2-ME</td>
<td>0.1 ml</td>
<td>100%</td>
</tr>
<tr>
<td>0.004% bromophenol blue</td>
<td>10 mg</td>
<td></td>
</tr>
</tbody>
</table>

Final volume was made up to 1 ml with distilled water.

**Electrode buffer for SDS PAGE:**

- Tris Buffer: 3.0g
- Glycine: 13.3g
- SDS: 1g

Dissolved and made the final volume to 1 liter in water.

Sodium phosphate mono basic buffer (0.2M): Dissolved 27.8g of sodium phosphate in 200 ml and final volume made up to 1000 ml with distilled water.

Sodium phosphate di-basic buffer (0.2 M): Dissolved 53.65g of dibasic sodium phosphate in 200 ml distilled water. Final volume made up to 1000 ml.

For pH 6.0, 87.7 ml mono-basic and 12.3 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

For pH 7.0, 39.0 ml mono-basic and 61.0 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

For pH 7.5, 16.0 ml mono-basic and 84.0 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

Acetate buffer:

0.2M solution of acetic acid: 11.55 ml of acetic acid dissolved in 1000 ml of distilled water.
0.2M solution of sodium acetate: 16.4g of sodium acetate dissolved in 200ml-distilled water and made up the final volume 1000 ml.

For pH 5.0, 14.8 ml of acetic acid and 35.2 ml of sodium acetate was mixed and made to final volume to 100 ml to get the buffer of 100 mM.

For pH 5.6, 4.8 ml of acetic acid and 45.2 ml of sodium acetate was mixed and made to final volume to 100 ml to get the buffer of 100 mM.