CHAPTER – 3
MATERIALS AND METHODS
The present work aimed at identification of proteins which are specifically altered (induced or suppressed) in response to drought stress was carried out at the Molecular Biology and Tissue Culture Laboratories of Jacob School of Biotechnology and Bioengineering, SHIATS, Allahabad.

3.1: Plant material:
Seeds of ‘Birsa Dhan-101,’ drought tolerance cultivar generated through pedigree method involving Fine gora and IET-2832 at Birsa Agricultural University, Ranchi and seeds of IR 20, drought susceptible generated through pedigree method involving IR 262-24-3 and TKM 6 at IRRI, were used for the present study as explants.

For this experiment following quantity of seeds were used:

A. Birsa Dhan 101 - 500 gm (Aprox 10 gm/tray)
B. IR 20 - 500 gm (Aprox 10 gm/tray)

The seeds of Birsa Dhan-101 were the kind gift from Dr. C.S. Mahto, Birsa Agricultural University, Ranchi and the seeds of IR 20 were the kind gift from Dr. A.K. Mall, Central Rice Research Institute, Cuttack, Orissa.

3.2: Surface sterilization of explants
The explants employed in this study were seeds of rice. The explants were thoroughly washed with tap water up to 3 to 4 times. Then they were treated with detergent for 10 minutes and again thoroughly washed with tap water. After this the explants were further treated with (30% v/v) sodium hypochlorite solution for 30 minutes. The surface sterilization conditions were optimized to minimize infection and tissue damage.

3.3: Inoculation of explants
The explants were inoculated in sterilized conditions in plastic trays on absorbent cotton and sterile distilled water was poured to different trays. Tolerant and susceptible varieties were inoculated separately. The explants were grown aseptically in the culture room conditions at 25°C ± 2°C under 1500 lux light intensity with 16 hr. photoperiod

3.4: Drought stress treatment
For drought stress treatments, 4 day-old germinated seedlings were used. Up to 4th day, watering was done every day. After 4 day of germination that is on 5th day, rice seedlings were subjected to drought stress for different time intervals (6, 12, 18, 24,
30, 36, 42, 48, 54, 60, 66, 72 and 96 hours) and after that seedlings were recovered for the same period in control condition.

Water deficit cycle namely 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 96 h stress and recovery along with control were used in this study. Controlled plants were watered everyday. Drought has been created according to the procedure of (Rizhsky et al., 2002) with some modifications. Drought stress was imposed by withdrawing water from plants until they reached a relative water contents (RWC) of 65% to 70%. After reaching the RWC of 65 to 70% drought stress was given for 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 96 h. Each tray was recovered for the same period of stress treatment. For example after 6 h of drought stress treatment, half plants were harvested and half were recovered for 6 h i.e. after 6 h of watering the rest of plants were harvested. The relative water contents were measured based on the method described by Turner (1981). The relative water content was determined in the fully expanded leaf. The fresh weights of the sample leaves were recorded, and the leaves were immersed in distilled water in a Petri dish. After 2 h, the leaves were removed, the surface water was blotted off, and the turgid weight was recorded. The samples were then dried in an oven at 70°C to constant weight. The relative water content was calculated using the following formula:

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

where FW is the fresh weight; DW is the dry weight; and TW is the turgid weight.

3.5: Collection of stressed and recovered samples

Roots and shoots of desired seedlings (starting from four days old seedlings) from each tray were collected after 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 96 hours of stress (in this case without water) and after 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 96 hours from recovery (that is after watering). Tolerant and susceptible varieties were harvested separately. They were then immediately frozen in liquid nitrogen and subsequently stored at −70°C for the protein extractions for electrophoresis.

3.6: Growth and recovery pattern studies

Four days old seedlings were subjected to 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 96 hours of drought stress and then subjected to recovery period of 72h in the
culture room (control conditions) in each case. The analysis was performed in duplicate set and average of the two sets was taken.

3.7: Extraction and analysis of soluble proteins
The parameters for the soluble protein analysis were optimized. Parameters such as protein load, staining, gel running conditions were optimized for obtaining good quality, reproducible protein gels.

3.8: Preparation of sample for one dimensional analysis
Samples for one dimensional analysis were prepared following the procedure of (Dubey et al. 2003). Shoots of rice seedlings were homogenized in liquid nitrogen up to the formation of fine powder. Polyvinyl poly pyrrolidone (PVPP) was added to the samples (50 mg/ g fresh weight of the tissue) during homogenization. The powder was then dissolved in Zivy buffer (Table 3.1). This extract was centrifuged twice at 10,000 rpm for 15 min at 4°C and the supernatant was transferred to centrifuge tubes. Then the soluble protein extract was precipitated in pre-chilled acetone and β-mercaptoethanol (0.07% v/v) solution overnight at –20°C. It was again centrifuged and the pellet, which contained the total protein, was dissolved in Laemmli buffer (Table 3.2). This solution was then heated in boiling water for 5 min. After centrifugation, required amount of supernatant was taken to estimate the protein concentration by Bradford (1976) method.

<table>
<thead>
<tr>
<th>Table 3.1: Composition of Zivy buffer (Zivy et al., 1983)</th>
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</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 8.8)</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
</tr>
<tr>
<td>EDTA-Na</td>
</tr>
<tr>
<td>DTT</td>
</tr>
<tr>
<td>MgCl₂</td>
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<td>PMSF</td>
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<tr>
<th>Table 3.2: Composition of Laemmli buffer (Laemmli, 1970)</th>
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<tbody>
<tr>
<td>Tris-HCl (pH 8.8)</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
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<tr>
<td>EDTA-Na</td>
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<tr>
<td>DTT</td>
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<tr>
<td>MgCl₂</td>
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<tr>
<td>PMSF</td>
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3.9: Preparation of sample for Two Dimensional analysis

In order to obtain good quality two dimensional gels, efforts were made to optimize the different parameters in the initial course of the study. The proteins were extracted essentially as per the protocol of (Dubey et al. 2003). Shoots of rice seedlings were homogenized in liquid Nitrogen up to the formation of fine powder. Polyvinyl poly pyrrolidone (PVPP) was added to the samples (50 mg/ g fresh weight of the tissue) during homogenization. The powder was then dissolved in Zivy buffer (Table 3.1). This extract was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was transferred to Eppendorf tubes. Then the soluble protein extract was precipitated in TCA for 1 h. It was again centrifuged at 14,000 rpm for 20 min at 4°C and the pellet, which contained the total protein, was washed with Acetone for 30 minutes. This solution was again centrifuged at 14,000 rpm for 10 min at 4°C and the pellet was dissolved in 150µL Reswelling buffer (Table 3.3) (Pandey et al., 2006). Suitable aliquots of the supernatant were used for performing quantification by utilizing Bradford dye.

Table 3.3: Composition of Reswelling buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>Urea</td>
<td>8 M</td>
</tr>
<tr>
<td>CHAPS</td>
<td>2% w/v</td>
</tr>
<tr>
<td>DTT</td>
<td>50 Mm</td>
</tr>
<tr>
<td>Bio-Lyte® 3/10Ampholytes</td>
<td>0.2%</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01% w/v</td>
</tr>
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</table>
3.10: Protein quantification

Amount of proteins in different extracts were estimated following the procedure of Bradford (1976). Throughout this study, proteins from the extract were precipitated by adding equal volume of 20% (w/v) TCA for 2 h (4°C), in order to minimize interference of chemicals such as Tris, 2-mercaptoethanol and SDS (Orr et al., 1988) in development of colour by Bradford dye. TCA-insoluble protein pellet obtained after centrifugation (15000xg, 5 min) was dissolved in 1 N NaOH (2 h, room temperature) and an aliquot of this solution was quantified for protein content. Standard calibration curve was made using bovine serum albumin (BSA) purified fraction IV (dissolved in 1 N NaOH).

3.11: One dimensional linear SDS-gel analysis of samples

For the analysis of high molecular weight proteins, procedure of (Dubey et al., 2003) was followed. A 7.5% linear vertical resolving gel of 1mm thickness (Table 3.4) and over it stacking gel (Table 3.5) was prepared. Then the gels were allowed to polymerize for about 1 h at room temperature and wells were prepared. Required quantity of the total soluble protein extract was loaded in the wells. Gels were run at constant current of 20 mA where the samples were initially in the stacking gel and at 30 mA the samples were in the resolving gel.

3.12: Conditions for running protein gels

SDS-gels were run at constant current of 20 mA per gel (10°C) till bromophenol blue dye reached the interface between stacking and resolving gel and at 30 mA per gel thereafter. In some instances, gels were over-run for 45 min to 1 h for increased separation of proteins.

Table 3.4: Composition of Resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tr>
<td>Tris-HCl (pH 8.8)</td>
<td>0.375 M</td>
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<tr>
<td>Acrylamide</td>
<td>7.5%</td>
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<tr>
<td>SDS</td>
<td>0.01%</td>
</tr>
<tr>
<td>APS</td>
<td>0.05%</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.06%</td>
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</table>
Table 3.5: Composition of Stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>0.125 M</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>3.9%</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
<tr>
<td>APS</td>
<td>0.135%</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.14%</td>
</tr>
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</table>

3.13: Two Dimensional gel electrophoresis: - Optimization of parameters of 2-D gels for Silver staining

The parameters for obtaining good quality silver stained two dimensional gels were optimized using various permutations and combinations of the protein extraction and gel running conditions. Varying amounts (50, 100, 150 and 164 µg) of total protein extract was loaded and eletrophoresed in the first dimension. The number of protein spots was found to significantly increase with the increasing protein concentration. The resolution of the two dimensional gels was found to be extremely sensitive and a large number of polypeptides (500-1000 in the silver stained gels) were observed with high resolution with almost negligible background in the gel.

While *de novo* synthesized proteins reflect proteins which have high turnover rates, steady-state proteins reflect the complete nature of cellular proteins. Steady-state proteins are likely to show those protein alterations also which have very low turnover rates. Therefore, analysis of steady state proteins was carried out in a separate analysis. The steady-state proteins were analyzed following silver staining.

Different amounts of the proteins were loaded as shown for individual experiments. Iso-electric focusing (IEF) of approximately 500µg of protein reswelling buffer was run using immobilized pH gradient strips (Ready Strip™ IPG Strips 7cm, pH 3-10, Catalog 163-2002, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547) for 12 h at 50 V, 15 min at 250 V, 2h gradient to 4000 V, and hold at 500 V
until the run is stopped. After IEF, IEF strips were equilibrated for 10 min in SDS-PAGE equilibration buffer I (pH 8.8) (6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol, 2% w/v DTT) followed by SDS-PAGE equilibration buffer II (pH 8.8) (6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol, 2.5% w/v iodoacetamide). Second dimension SDS-PAGE was run in BioRad Protein® x Cell according to manufacturer recommendations. Gels were transferred in fixation solution (methanol: acetic acid: water, 40:10:50 v/v) for 10 min followed by silver staining. The gels were then photographed and spots were compared.

3.14: Silver staining

In order to get total profile of protein, silver staining was done following the procedure of (Dubey et al., 2003). For this purpose, the gels were left overnight in the fixative solution containing 40% (v/v) methanol, 10% (v/v) acetic acid, and 50% (v/v) water. The gels were then washed in methanol and were repeatedly rinsed with Na₂CO₃. Then they were treated with Farmer reducer containing 0.15% w/v potassium ferrocyanide, 0.05% w/v Na₂CO₃ and 0.3% w/v sodium thiosulphate. Then they were subsequently rinsed with distilled water and subjected to 0.1% AgNO₃ treatment. The gels were again treated with Na₂CO₃ and then developed by using the developer solution (2.5% Na₂CO₃ and 0.02% HCHO) until clear bands were obtained. Then the process was terminated by the use of 5% (v/v) glacial acetic acid solution. The various steps involved in this protocol are shown in the form of flow sheet below.

As mentioned above, parameters for obtaining good quality silver two dimensional gels using various permutations and combinations of the protein extraction and gel running conditions were optimized in the course of this study. With regards to silver staining, varying amounts (50, 100, 150 and 164 μg) of total protein extract was loaded and electrophoresed in the first dimension. The number of protein spots was found to dramatically increase with the increasing protein concentration. However, an overloading effect started to emerge when the concentration was raised above 100 μg.
This phenomenon was highlighted by the gradual shift of protein spots towards the basic end coupled with the deterioration in the resolution of the protein spots.