Chapter 3

Materials and Methods
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MATERIALS AND METHODS

Eight saline resistant varieties of rice (*Oryza sativa* L.) released from Mankombu Rice Research station was selected for studying the saline tolerant characteristics in the pilot study of the present investigation. They were MO5, MO6, MO7, MO8, MO9, MO10, MO18, and MO19. After preliminary germination studies, two varieties, MO18 and MO19 were found to be more tolerant and selected for further investigation. MO18 called “Krishna” (100 seeds equals 2.45 gm - Fig. 1) was released from Moncombu in 1998 and has a life span of 115 to 120 days. This is a high yielding variety produced by hybridization between MO1 x MO6. MO19 “Krishananajana” (100 seed equals 2.78 gm - Fig. 2) was released in 1998, obtained from a cross between MO1 and MO6 has a life span of 105 to 110 days.

3.1. Morphological Parameters
3.1.1. Germination Studies
Seeds were surface sterilized with 0.1 % mercuric chloride solution and placed on petridishes (15cm diameter) lined with Whatman No.1filter paper and moistened with NaCl solution of different concentrations (0.1, 0.3, 0.5, 0.7, and 0.9 per cent). 40 seeds after 24 hour soaking in distilled water prior to NaCl treatment were used for each treatment. A control, free from NaCl treatment was also maintained. All germination tests were carried out with 12hrs light period (Sylvania cool white fluorescent lamps 25μ mol m^-2 s^-1, 400-750nm) at 25 ± 2°C. The number of germinated seeds was counted and the percentage was calculated by following formula.

\[
\text{Germination Percentage} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100
\]

3.1.2. Root Characteristics
In order to study, the root characteristics in controlled and saline conditions, the seedlings were grown in nutrient solution (Hoagland and Arnon, 1938) in culture tubes.

3.1.2.1. Seminal Root Length
The seminal root length was measured on 18th day after germination. Seedlings were washed in water and the root length was measured in cm using a scale and thread. 10 observations were made from randomly selected samples form each treatment and the average was found out.
3.1.2.2. Number of Nodal Roots
After 18 days of growth the numbers of nodal roots were counted from 10 plants from each treatment (Dittmer, 1948) and the average of a single seedling was found out.

3.1.2.3. Total Length of Nodal Roots
Total length of the nodal roots could be measured in mm using a scale. Average of ten observations from each treatment was taken and the average of a single plant was found out.

3.1.2.4. Total Number of Root Hairs per Unit Length of Nodal Roots
Number of root hairs on the nodal roots was counted per mm under the low power view of the microscope from 10 randomly selected nodal roots from each treatment and control (Dittmer, 1949)

3.1.2.5. Length of Root Hairs
Length of 10 root hairs from random selection was measured from each treatment with the help of an ocular micrometer and the average length was found out.

3.1.2.6. Stereo Microscopic Study of Root Hair
The MO18 and MO19 varieties were allowed to germinate in petridishes under the conditions such as 0.0 per cent, 0.5 per cent and 0.9 per cent NaCl. After two days of germination, the roots were fixed in a mixture of 4 per cent formaldehyde + 70 per cent alcohol + 1 per cent acetic acid. The temporary slides were prepared and root hairs were observed under the Stereomicroscope (Leica MSS) and photos were taken with digital camera (Canon Power Shot S40).

3.1.2.7. Scanning Electron Microscopic Study of the Root Tip
Root tips from treated and control seedlings were fixed in a mixture of 4 per cent formaldehyde + 70 per cent alcohol + 1 per cent acetic acid and dehydrated. Further treatment was completed in ethanol-amyl acetate grades (i.e. 1:1,100%), samples were air dried and coated with silver by using a vacuum unit (Model, Nano-Tech, Manchester), scanned and photographed by using Cambridge S4-10 Scanning Electron Microscope.

3.1.3. Shoot Characteristics
For obtaining shoot characteristics, seeds of MO18 and MO19 were grown in controlled and saline conditions, the seedlings were grown in nutrient solution (Hoagland and Arnon, 1938) in culture tubes.
3.1.3.1. Length of Shoot
Shoot length was measured in cm from 18 day old plants at random from each treatment and control.

3.1.3.2. Leaf Area
Leaf area of randomly selected plants from each treatment and control were measured on 45th day after germination using image J software with the help of flat bed scanner.

3.1.3.3. Biomass

**Fresh Weight**
Fresh weight was taken after uprooting the entire plant without any damage, washing with water and wrapping the seedlings in muslin and additional mild centrifugation by use of a low-speed bench centrifuge. (Linford and Rhoades, 1989). The roots were weighed separately for each treatment and control in an electronic balance (Dhona Electrical Balance). The data were analyzed and mean was taken from ten observations from each treatment.

**Dry Weight**
Dry weight was determined from the washed seedlings after drying at $70^\circ$ C for 48 hours in an oven until a constant weight was obtained (Schuurman and Goedewaagen, 1971). Relative water content was estimated as the difference between dry weight and fresh weight.

3.2. Physiological Parameters

For obtaining physiological parameters seedlings were grown in nutrient solution (Hoagland and Arnon, 1938) in culture tubes. Fresh samples of both the control and under treatment were taken for the analysis of physiological parameters.

3.2.1. Ions

3.2.1.1. Extraction of N, P, K and Cl$^-$ ions
The procedure of Wignarajah et al., (1975) was employed for the extraction of ions. Known weight of (100mg) dry powdered plant material was extracted thrice with boiling deionized water and the supernatant was collected after centrifugation at 6,000 rpm. for 10 minutes. The residue obtained was re-extracted with 30% (V/V) nitric acid at $90^\circ$ C for one hour. The suspension was cooled and the supernatant was collected by centrifugation at 6,000 rpm. for 10 minutes. The nitric acid extraction procedure was repeated thrice. All the supernatants were pooled and made up to a known volume (25ml). Na$^+$, K$^+$, P and Cl$^-$ ions were estimated flame photometrically, (Chemito 1020, India). Chloride was determined by titration against silver nitrate using potassium chromate as indicator, (Clesari et al., 1989). The quantity of
AgNO₃ required for colour development was recorded and chloride content of solution was calculated using the following formulae,

\[ \text{mg Cl}^-/\text{L} = \frac{(A - B) \times N \times 35}{\text{ml sample}} \]

Where

- \( A = \) ml titration for sample
- \( B = \) ml titration for blank
- \( N = \) Normality of AgNO₃.

### 3.2.2. Chlorophyll Content

Total chlorophyll content was estimated using the method proposed by Arnon, (1949). Weighed out 1gm of finely cut and well mixed representative sample of leaf was ground to fine pulp with the addition of 20 ml of 80 per cent acetone. Centrifuged at 5,000 rpm. for 5 minutes and transferred the supernatant to a 100 ml volumetric flask. Ground the residue with 20ml of 80 per cent acetone, centrifuged and transferred the supernatant to the same volumetric flask. Repeated this procedure until the residue was colourless. Washed the mortar and pestle thoroughly with 80 per cent acetone and collected the clear washings in the volumetric flask. Made up three volumes to 100 ml with 80 percent acetone. Read the absorbance of the solution at 645 and 663 nm against solvent blank.

\[ \text{mg total chlorophyll /g tissue} = 20.2(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000} \times W \]

Where

- \( A = \) absorbance at specific wave length
- \( V = \) final volume of chlorophyll extraction 80 Percent acetone
- \( W = \) fresh weight of the tissue extracted

### 3.3. Biochemical Parameters

For obtaining biochemical parameters seeds of MO18 and MO19 were sown in vermiculite and sand mixed in a ratio of 3:1 and grown in controlled growth chamber set at 25°C and 75% RH with a 16hrs auto period. Each variety was grown separately and seedlings were watered three times a week. ½ MS medium is applied to the vermiculite and sand on alternate days. On the 14th day after sowing NaCl solution of different concentrations were applied to
the seedlings. After 4 days of stress, the samples were collected for studying different biochemical parameters

3.3.1. Starch

100mg. of plant material was ground in 80% ethanol and extracted twice. The residue was dissolved in known volume (20ml.) of 0.7% KOH and it was gelatinized in boiling water bath for 40 minutes. It was cooled and made up to a known volume (Chinoy, 1939). Known volume of this aliquot was mixed with 6ml. of iodine solution (2.5g I₂ and 25g KI in one litre of 0.05N HCl). The OD was read at 600nm. The following regression formula was prepared by using known concentration of starch and the starch content was expressed as mg g⁻¹ DW.

\[ C = K \times OD + B \]

Where

- \( C = \) concentration
- \( K = 347.82 \)
- \( B = -5.0156 \)

3.3.2. Protein

The method of Lowry et al., (1951) was used for protein extraction and estimation. Fixed weight of plant material was ground in 80% ethanol and extracted twice. The residue was washed first with 5% HClO₄ and secondly with a mixture of ethanol: ether: and chloroform (2:1:1) to remove acid soluble fractions and lipids. The residue was then washed with 1M cold trichloroacetic acid (TCA) and centrifuged. The protein fraction was dissolved in 1N sodium hydroxide for one hour and centrifuged. The supernatant was made up to a known volume (5ml) and used as aliquot. To a known volume of aliquot 5ml of reagent C prepared by mixing 50ml of reagent A (2% sodium carbonate in 0.1N NaOH) and 1ml of reagent B (0.5% cupric sulphate in 1% sodium potassium tartarate) was added and incubated for 10 minutes at room temperature. The colour was developed by adding 5ml of 1N Folin phenol reagent and incubating for 30 minutes at room temperature. The O.D. was read at 725nm. The following regression formula was prepared by using known concentrations of Bovine serum albumin (BSA) and protein concentration was expressed as protein g⁻¹ DW.
\[ C = K \times OD + B \]

Where

- \( C \) = concentration,
- \( K = 374.04 \)
- \( B = -7.2020 \)

### 3.3.3. Amino acid

The content of free amino acids was determined following the method of Harding and Mc Clean (1916). The reaction mixture contained 0.5ml of ethanol extract of material, 1ml of 10% Pyridine and 1ml of 2% ninhydrin- reagent. The test tubes were stopped and heated at 100 \( ^\circ \)C on a water bath for 30 minutes, cooled and diluted with distilled water to a known volume (25ml). The O.D. of the violet colour developed was read at 570nM. The following regression formula was prepared by using isoleucine as a standard and the amino acid content was expressed as mg g\(^{-1}\) D.W.

\[ C = K \times OD + B \]

Where

- \( C \) = concentration
- \( K = 117.86 \)
- \( B = 5.5226 \)

### 3.3.4. Proline

Proline was extracted and estimated according to the method described by Bates et al., (1973). 200mg. of fresh plant material was homogenized in 4 ml 3% (w/v) aqueous sulfosalicylic acid and the homogenate was filtered through Whatman No 1 filter paper. 2ml of the filtrate was reacted with 2ml of acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10ml of 6 M orthophosphoric acid) and 2ml glacial acetic acid in a test tube at 100 \( ^\circ \)C for one hour and the reaction was terminated by keeping the tubes in ice bath. The reaction mixture was extracted with 6ml toluene in a separating funnel. The absorbance was read at 520 nm against a toluene blank. Proline concentration of the extract was determined from the following regression formula described above for amino acids using proline as standard and expressed as \( \mu g \) g\(^{-1}\) FW.
C = K \times \text{OD} + B

Where

\begin{align*}
C &= \text{Concentration} \\
K &= 30.19 \\
B &= -2.7550.
\end{align*}

3.3.5. **Enzyme**

Known weight (0.5g) of plant material was thoroughly ground in a cold mortar with pestle in an ice-bath, until no fibrous residue could be seen. The grinding medium (4-6ml fresh weight) consisted of 100mM phosphate buffer (pH 7.8) and 0.1mM EDTA, 1% (w/v) Polyvinylpyrrolidone plus homogenizing glass beads. The homogenate was centrifuged, twice at 13,000g for 10 minutes at 0 to 4°C in a refrigerated centrifuge (Beckinan-Avanti model U.S.A). The supernatants were pooled and made up to final volume (10ml). It is hereafter referred to as crude enzyme extract, and was used for isoenzyme analysis as well as for total activity assays. An aliquot of the extract was used for protein determination (Lowry et al., 1951).

3.3.5.1. **Peroxidase Assay**

Peroxidase-activity was measured according to the method of Shannon et al., (1966). The assay mixture consisted of 2.8ml of O-dianisidine buffer, 0.1ml H₂O₂ (1% w/v) and 0.1ml enzyme extract. The absorption of resulting solution was recorded spectrophotometrically (Shimadzu-UV-160) at 460nm at every 30 sec intervals up to 90sec. The amount of enzyme required to change the absorption (\Delta OD) by 0.001min⁻¹mg⁻¹ protein was taken as unit enzyme activity.

**Superoxide Dismutase assay**

The activity of Superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The 3ml of reaction mixture contained 50µM phosphate buffer pH 7.8), 13µM methionine, 75µm NBT, 2m riboflavin, 0.1µM EDTA and 0.1ml enzyme extract. Riboflavin was added last and the tubes were shaken and placed to 30cm below a light bank consisting of two 15W white fluorescent lamps. The reaction was started by switching on the light and was allowed to react for 10 minutes during which time it was found earlier to be linear. The reaction was stopped by switching off the light and the tubes were covered with a Black cloth. The absorbance of the reaction mixture was read at 560nm. A non irradiated
reaction mixture did not develop colour and served as control. There was no measurable
effect of the diffused daylight. The reaction mixture lacking enzyme developed maximum
colour and colour intensity decreased with increasing volume of enzyme extract added. Log
A 560 was plotted as function of the volume of the enzyme extract used in the reaction
mixture. (Giannopolitis and Ries, 1977). From the resultant graph the volume of the enzyme
extract corresponding to 50% inhibition of the reaction was read and was considered as one
enzyme unit (Beauchamp and Fridovich, 1971). Specific activity was expressed as unit mg^{-1}
protein.

3.3.6. Electrophoretic Separation of Proteins

3.3.6.1. Protein

The method of Boothe et al., (1995) was employed for extraction and separation of proteins
using electrophoresis. Leaves were frozen in liquid nitrogen and ground in mortar with a
pestle to fine power. Protein extraction buffer 5ml g^{-1} fresh weight tissue (50mM Tris HCl,
pH 8.0, 2% (w/v) SDS, 1mM PMSF, 10% (v/v) glycerol) was added, and grinding was
continued till the tissue was thoroughly homogenized. The soluble proteins extracted in the
same buffer without SDS. The extracts were centrifuged (10, 000g. for 10 minutes at 0 – 4^0
C) to remove insoluble debris. Protein concentration was determined according to the method
of Lowry et al., (1951).

**SDS – PAGE**

One dimensional gel electrophoresis was carried out according to the procedure of Laemmli
(1970) on 12.5% (w/v) acrylamide slab gels (1.5mm thick). The running gel contained 1%
(w/v) SDS, 0.378M Tris – HCl, (pH 8.8), 20% acrylamide, 0.7 % bisacrylamide, 0.35%
ammonium persulphate and 10μl TEMED (N, N', N'' Tetramethylethylene diamine). The
stacking gel contained 0.125M Tris-HCl (pH6.8), 3.75% acrylamide, 0.135% bisacrylamide,
0.75% ammonium persulphate and 15μl of TEMED. The electrode buffer contained 20mM
Tris Glycine (pH 8.3) and SDS (0.1w/v). Prior to electrophoresis, an equal volume of loading
buffer (100mM Tris-HCl, pH 8.0, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v)
glycerol, 0.025% (w/v) bromophenol blue) was added to each sample (containing150-200 μg
protein) and the samples were boiled at 100^0C for 2 minutes. The gel were run at a constant
current of 60mA for 4 hours at 4^0C and stained with silver nitrate and photographed. Known
molecular weight markers (Sigma) were used as standards for calculating the molecular
weight.
3.3.6.2. Native Polyacrylamide Gel Electrophoresis (Isoenzyme Study)

Slab gel electrophoresis was performed to separate the isoenzymes of peroxidase (POD, E.C.1.11.1.7) and Superoxide dismutase (SOD, EC. 1. 15.1.1) in a system of continuous running gel (7.5% acrylamide + 0.2% bisacrylamide) overlaid by a stacking gel (4.5% acrylamide + 0.12% bisacrylamide), Cavalcante et al., (1994). Each well was loaded with 20μl of sample extract. The electrode buffer was composed of 4.8mM Tris and 38mM glycine (pH 8.3). The gels were run for 3hours at 4°C at a constant current of 30mA. The migration front was followed with bromophenol blue. The relative mobility of each enzyme was established with reference to the position of tracker dye by dividing the distance travelled by that isoenzyme by the distance travelled by the tracker dye.

Staining of the Gels

After the completion of electrophoresis, the gels were stained for isoenzymes of POD and SOD following the procedures of Gulati (1989), Misra and Fridovich (1977) and Sako and Stahmann (1972) respectively.

Peroxidase (POD)

To localize the peroxidase the gels were incubated in a mixture of 0.6ml guaiacol and 0.5ml 3% H₂O₂ (added just before staining) in 100ml of 0.2M acetate buffer (pH4) at 26°C for 15-20 minutes.

Superoxide Dismutase (SOD)

Native gels were stained for SOD activity by incubating in a solution containing 2.5mM nitroblue tetrazolium for 25 minutes, followed by incubation in 50mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28mM tetramethyl ethylene diamine (EDTA) for 20 minutes in dark. The gels were placed in distilled water and exposed to light for 10 to 15 minutes at room temperature to visualize isoenzymes.

3.4. Anatomy

3.4.1. Leaf (TEM) for Chloroplast and Mitochondria

The middle sections of 2nd leaves (1-2mm) showed no visible symptoms, were fixed in Karnovski's fixative (mixture of 4% paraformaldehyde and 5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2). To prevent the entry of air bubbles, the sections were cut with a sharp blade keeping them immersed in their respective fixatives. The leaf sections were fixed at room temperature for 4 hrs. and subsequently rinsed with 0.05M phosphate buffer, pH 7.2, 6 times at 30-minute intervals. Tissues were then post fixed in 2% OsO₄ in 0.05 M phosphate
buffer, pH 7.2, at 4°C for 12 hrs. and dehydrated with a graded series of acetone (30, 50, 70, 90, 99, and 100%) and finally acetone was replaced by propylene oxide. The samples were then embedded in Spur resin (Spurr, 1969) at room temperature and polymerized in an oven at 70°C for 24 hrs.

Semithin sections (1µm in thickness) were cut with glass knives and stained with 0.5% toluidine blue and 1.5% Na₂CO₃ on a heating plate (60°C). Then the sections were observed under a light microscope (Nikon optihot-2). For electron microscopy, ultrathin sections (70-90µm in thickness) were cut with a diamond knife and placed on 200 mesh copper grids. The grids were stained with 2% uranyl acetate for 20 min followed by lead citrate for 5 min. Then the sections were viewed at 100 kV on a Hitachi H600 transmission electron microscope (TEM). Four thin sections from different leaves and roots per treatment were examined. Photographs were taken at three or more random sites in different sections and representative pictures are presented. The number of mitochondria per cell section was calculated in more than five cells from different individuals per treatment.

3.4.2. Root

Root structures were examined with the help of longitudinal sections of 3µ in thickness cut with the help of a freezing microtome. Several positions in the root tip and in the root cap were observed and good slides were photographed.

Statistical Analysis

The data recorded on various parameters were subjected to analysis. The treatments were evaluated with reference to each parameter by estimating mean, range, standard deviation and coefficient of deviation. Analysis of variations conducted for certain characters and the format table given below.

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