Chapter II

MATERIALS AND METHODS

1. Materials

Seeds of the following varieties of ragi were used for the present investigation :

(1) Indaf-1 (2) Indaf-8 (3) Indaf-10 (4) Indaf-11
(5) Purna (6) PR-202 (7) HR-374 (8) JNR-981-1
(9) HBP 7-6 (10) CO-7 and (11) CO-11.

These seeds were obtained through the courtesy of different agricultural research stations in India.

2. Methods

A. Germination Studies

Healthy seeds of ragi were sorted out and surface sterilized with 0.1 % $HgCl_2$ solution for 5 minutes. After 5 minutes of soaking in $HgCl_2$ solution, the seeds were washed thoroughly and air dried. Twenty five seeds were kept in sterilized pettidishes for germination over Whatman No.1 filter paper. The filter paper was moistened with 15 ml of distilled water. The experiment was carried out (in triplicate) at 28°C in germination chamber for 120 hours (5 days). The emergence of radicle from seed coat was acknowledged as a criterion for germination counts.

In order to have a preliminary idea about the metabolism of ragi seeds during germination at different stages of germination the analysis of seedlings were made with respect to moisture percentage, soluble sugars and enzymes like

α-amylase, acid phosphatase, alkaline phosphatase, dehydrogenase, peroxidase and catalase. The moisture percentage was determined by subjecting a known amount (wt.) of seedling to high temperature (60°C) in oven until a constant weight is obtained and noticing the difference in these weights.

The soluble sugars in the acid hydrolized alcohol extract of seedling were determined by following the method of Nelson (1944).

For the study of enzyme α-amylase (EC.3.2.1.1) a modified blue value method of Katsumi and Fukuhara (1969) was followed. For this 0.5 g of different stages of germinating seeds were crushed in a chilled mortar with pestle in 10 ml cold acetate buffer (pH 5) and filtered through four layers of cheese cloth. The filterate was centrifuged at 10,000 g for 20 minutes and the supernatant was used as the enzyme source. Two sets of mixtures were prepared one serving as '0' min. control and the other serving as reaction mixture. One ml of acetate buffer (pH 5) and one ml of enzyme solution were mixed in a test tube. After a 10 minutes of equilibration period one ml of .2 % amyloze solution (200 mg of amylose were dissolved in 4 ml of 1 N NaOH and kept in a refrigerator over night to dissolve completely. Then it was diluted to 80 ml with distilled water and adjusted to pH 7.5 with 1 N acetic acid and brought to a final volume of 100 ml with the addition of distilled water) was added. The reaction in control ('0' min)

set was terminated immediately following the addition of substrate by adding 10 ml of 0.5 N acetic acid. The reaction mixture in other set was shaken for exactly 30 minutes and after this period the reaction was terminated by adding 10 ml of 0.5 N acetic acid. One ml of each reaction mixture was sampled and mixed with 10 ml of I_2KI solution. The O.D. of the solution was read at 700 nm against I_2KI solution used as blank. The enzyme activity was expressed as change in O.D./ min./g fresh wt.

For the study of enzyme acid phosphatase (EC 3.1.3.2) the method was adapted from the procedure described by De Leo and Sacher (1970) and McLachlan (1980). For the same, 0.5 g of seedling tissues were macerated by grinding in cold acetate buffer (pH 5) in an ice cooled glass mortar and pestle. The resulting suspension was filtered through four layers of cheese cloth and the filtrate was centrifuged at 10,000 g. A suitable aliquot of the supernatant was used in the assay. The assay mixture used was contained 3 ml of P-nitrophenyl phosphate (0.1 mg P-nitrophenyl phosphate/ ml of acetate buffer pH 5), 2 ml of acetate buffer (pH 5) and 1 ml of enzyme. The reaction took place at room temperature for 30 minutes and was stopped by the addition of 1.5 ml of 1.68 N NaOH. The reaction terminated immediately (0 Min) served as control. The optical densities of the developed colour were determined at 420 nm. The enzyme activity is expressed as change in optical density/ hr/g fresh wt.

The tetrazolium method of Kittech et al.,(1968) was followed for the estimation of dehydrogenase (E.C.1.1.1.a) in the different stages of ragi seed germination. 50 embryos were carefully isolated from seedlings of different stages with the help of forceps and needle. Then the embryos were incubated for 4 hours in 0.2 % 2-3-5 triphenyl tetrazolium chloride, solution in dark. After this the embryos were washed well with distilled water, surface dried and covered with 5 ml of 2-methoxy ethanol for extraction of the red coloured formazon. The optical density of the coloured formazon was recorded at 470 nm. The enzyme activity is expressed as O.D/Seedling.

For the study of enzyme peroxidase the (EC 1.1 .1.7) the method employed was of Machly (1954). Enzyme was extracted in cold distilled water by homogenizing 0.5 g of ragi seed, of different germination stages in cold mortar with pestle. Filtered through moist chees cloth (four layers) and the filtrate was centrifuged at 10,000 g for 20 min and the supernatant used as enzyme source. Enzyme assay mix was prepared by adding 2 ml of phosphate buffer (pH 7, 0.1 M), 1 ml of guiacol (20 mM) and 1 ml of enzyme. The reaction was started by the addition of 0.04 ml of H_2O_2 (10 mM). Change in optical density due to oxidation of guiacol was recorded per minute at 470 nm on colorimeter with frequent stirring of the reaction mixture with glass rod. Activity of enzyme is expressed as change in $O.D.min^{-1}g^{-1}$ fresh wt.

Catalase : (EC 1.11.1.6) was studied by following the method of Herberts (1955). For this 0.5 g of different stages of germinating seeds were crushed in chilled mortar with pestle in 10 ml of cold distilled water, filtered through four layers of cheese cloth. The filtrate was centrifuged at 10,000 g for 20 minutes in refrigerated centrifuge and the supernatant was stored at 0°C and used as the enzyme source. The assay mixture was prepared by mixing 1 ml of H_2O_2 (0.045 M) and 1 ml of enzyme. Assay mixture was incubated at room temperature for 1 minute and after that the reaction was terminated by the addition of 3 ml of H_2SO_4 (5 N). Then to this 1 ml of KI(10%) solution and a drop of Ammonium molibdate were added. The amount of H_2O_2 utilized by liberation of iodin was determined by titrating the reaction mixture with 0.01 M sodium thiosulphate using starch indicator. The difference between 0 and 1 min reaction was used as enzyme activity. For 0 min. reaction and blank reaction mixture was the same as in case enzyme assay mixture but for blank without enzyme and for 0 min. reaction was terminated immediately after or before the addition of enzyme. Activity of enzyme is expressed as H_2O_2 broken $min^{-1}g^{-1}$ of fresh wt.

For the study of enzyme alkaline phosphatase (EC 3.1.3.1) the method was adopted from the procedure described by Weimberg (1970). For the same 0.5 g of seedlings tissues were macerated by grinding in cold Tris-chloride buffer pH 8.0 containing 1 M KCl, 0.01 M EDTA (sodium salt) (pH 8.0) and 0.4 % β mecraptoethenol in an ice cooled glass mortar and

pestle. The resulting suspension was filtered through four layer cheese cloth and the filterate was centrifuged at 10,000 g in refrigerated centrifuge. A suitable aliquot of the superlatent was used in assay. The reaction mixture contained 1 ml 0.1 M tris-chloride buffer pH (7.5), 0.1 ml of 0.05 M $MgCl_2$, 1.7 ml H_2O, 0.2 ml enzyme extract, and 0.1 ml 0.02 M o-nitrophenylphosphate. The reaction was incubated for 30 minutes at room temperature. The optical densities of the assay mixture were measured at 0 min and 30 min. at 420 nm. The assay mixture without enzyme served as blank. The enzyme activity is expressed as change in optical density/ /g fresh wt..

B. Studies of germination under stress conditions

The influence of various environmental stresses on germination of ragi were studied by imposing different types of stress on germinating seeds.

a) Salinity tolerance studies

An extensive work on salt tolerance of rice at International Rice Research Institute has shown that the salt concentrations (0.4 % W/V) gives an ECe of approximately 8-10 mm hos/cm at 25°C and this can be considered as the discriminating level of salinity (Ponnamperuma,1977). Hence, for the present studies this level and a higher level (15 mm hos/cm) of sodium chloride salinity were chosen.

Healthy seeds of 11 varieties of ragi were sorted out and surface sterilized with 0.1 % $HgCl_2$ solution for 5 minutes.

They were washed thoroughly with distilled water and air dried. Twentyfive seeds were kept in sterilized petridishes for germination over Whatman No.1 filter paper. The filter paper was moistened with 15 ml of distilled water (control) or salt solution. The experiment was carried out (in triplicate) at 28°C in germination chamber for 120 hours (5 days). The emergence of radicle from the seed coat was acknowledged as a criteria for germination and germination percentage in every treatment was recorded after 24 hours interval. After 120 hours the seedling growth with respect to root length, shoot length, fresh weight and dry weight was recorded and statistically analysed by determining standard deviation.

The seeds of ragi variety HR-374 were used for the study of the behaviour of enzyme α-amylase, acid phosphatase, dehydrogenase, peroxidase and catalase during germination under saline conditions. The methods followed for the study of these enzymes are essentially similar to those described earlier.

b) Water stress

For imposing water (osmotic) stress, polyethylene glycol (PEG) with molecular weight 6000 was employed. The osmotic potentials - 5 bars and - 10 bars of aqueous solution of PEG were selected and these were adjusted according to the method described by Michel and Kaufmann (1973). The germination studies of eleven ragi varieties were performed as described under salinity tolerance.

The seeds of ragi variety HR-374 were used for studying the behaviour of enzymes α-amylase, acid phosphatase, dehydrogenase, peroxidase and catalase during germination under water stress conditions. For these studies also two osmotic potentials (-5 and -10 bars) of PEG were employed and enzymatic studies were performed according to the methods described earlier.

c) Boron tolerance studies

For studying the effect of boron on germination and varietal responses to boron toxicity, two concentrations of boron (25 ppm and 50 ppm) were chosen as recommended and practiced by Mehta (1970). Effect of these treatments on the developmental pattern of α-amylase, acid phosphatase, dehydrogenase, peroxidase and catalase were also investigated. The procedure followed for germination and enzymatic studies is essentially similar to that described earlier.

All experiments including enzymatic studies were performed in triplicate and mean of three determinations were regarded as final results in order to represent them graphically.