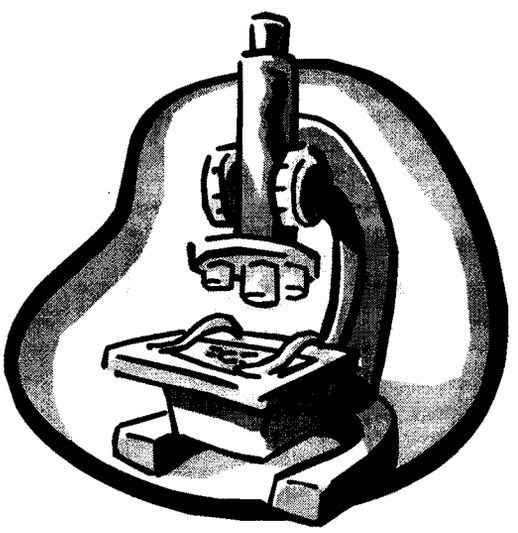

# CHAPTER II

# MATERIALS & METHODS

## A] GERMINATION STUDIES:

### a) Germination and seedling growth:

Seeds of wheat cv. Raj-1555 and moong Pusa-Baisakhi were obtained from authentic source in local market and from Agriculture College, Kolhapur. The viability of seed sample was tested first and then seed lots were employed for the investigation. To study influence of salicylic acid on various physiological parameters during germination seeds were surface sterilized with .1% $HgCl_2$ solution for 2 minutes. Then seeds were washed thoroughly several times with distilled water and blotted to dry. Twenty seeds were kept in each treatment in sterilized petriplates over Whatman paper no. one filter paper at room temperature in germination chamber. The filter paper was moistened with 10 ml of glass-distilled water in control and 5, 10, 50, 100 and 200-ppm salicylic acid solution in respective petriplates. Water uptake, respiration rate, activities of various enzymes and fate of various organic constituents were studied after 24 hours of seed germination in both wheat and moong seeds. For study of effect of SA on seedling growth 120 h old healthy seedlings from different treatments were employed.

### b) Water Uptake:

After 24 h germination 10 seeds from each treatment were carefully removed blotted to dry and their fresh weight was recorded. Then the seeds were kept in oven at $60^0$C. till a constant weight (dry weight) was obtained. The difference in these two weights was considered as an amount of water taken by seeds during germination.

**c) Respiration Rate:**

Two grams of seeds from each treatment were transferred to a small vial, which was hold carefully in a jar containing 2 ml 0.025 N barium hydroxide solution. The jar was properly sealed and made air tight, simultaneously another jar (control) containing 2 ml 0.025 N barium hydroxide solution but without seed material was also sealed and made air tight. The experimental set up was placed in dark for 60 minutes. After 60 minutes the vials with seed material were removed carefully from jar and the residual barium hydroxide solution was titrated against 0.025 N Hydrochloric acid using phenolphthalein as an indicator. The barium hydroxide solution in the control jar was also titrated in similar manner. The difference in titer values gives a measure of respiratory $CO_2$ evolution. The rate of respiration was calculated according to formula given by Mclean and Ivimey-Cook (1968) as mg $CO_2$ liberated $h^{-1}g^{-1}$ seed material.

Formula:-

$$\frac{\left\{\begin{array}{l}\text{Control titration} \\ \text{reading}\end{array} - \begin{array}{l}\text{Experimental titration} \\ \text{reading}\end{array}\right\} \times 0.22 \times 50 \times 60}{\text{Experimental time in minutes x weight of material in g}}$$

**d) Enzyme Dehydrogenase: (EC. 1.1.1.4)**

For the study of dehydrogenase from germinating wheat and moong seeds the tetrazolium method of Kittoch and Law (1957) was followed. The seed coat was removed from germinating seeds and one g of seeds were fragmented into small pieces and incubated for 4 hours in 4 ml 0.2% 2-3-5, Triphenyl tetrazolium chloride (TTC)

solution in dark. After this seedlings were washed 2-3 times with distilled water, surface dried and were treated with 10 ml of 2, methoxyethanol for extraction of red coloured formazan, which is formed due to activity of dehydrogenase. The optical density of coloured formazan was measured at 470 nm. The enzyme activity is expressed as change in OD $h^{-1}g^{-1}$ fresh weight.

**e) Enzyme α-amylase (EC. 3.2.1.1)**

A blue valve method of Katsumi and Fukuhara (1969) was followed for study of enzyme α-amylase. The seed coat was removed (in case of moong) 0.5 g of germinating seeds were crushed in a chilled mortar with pestle in 10 ml of cold acetate buffer (pH 5) and filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as enzyme source. Two sets of reaction mixture were prepared one serving as '0' minute control and other serving as a reaction mixture. One ml of acetate buffer (pH 5) and one ml enzyme solution were mixed in a test tube. After 10 minutes of equilibration period one ml of 0.2% amylose solution (200 mg of amylose were dissolved in 4 ml of 1 N NaOH and kept in refrigerator overnight to dissolve completely. Then it was diluted to 80 ml with distilled water and adjusted to pH 7.5 with 1N acetic acid and brought to final volume 100 ml with the addition of distilled water.) The reaction in control (0 minute) set was terminated immediately following the addition of substrate by adding 10 ml of 0.5 N acetic acid. The reaction mixture in another set was shaken for exactly 30 minutes and then the reaction was terminated by adding 10 ml of 0.5 N acetic acid. One ml of each

reaction mixture was taken in another set of test tubes and mixed with 10 ml of dilute $I_2KI$ solution. The change in OD of the solution was measured at 700 nm against $I_2KI$ solution used as blank. The enzyme activity was expressed as change in OD $min^{-1}mg^{-1}$ protein.

The soluble proteins in the enzyme extract were estimated following the method of Lowry *et al.*, (1951), which is described in details in latter part of this chapter.

**f) Enzyme acid Phosphatase [Apase] (EC. 3.1.3.2)**

Enzyme acid phosphatase was assayed according to the method of McLachlan (1980). 0.5 g of germinating seed material from each treatment was homogenized separately in 10 ml of cold acetate buffer (pH 5) in ice-cold mortar with pestle. The resultant homogenate was filtered through four layers of cheesecloth and filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source.

The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg p-nitrophenyl phosphate $ml^{-1}$ of acetate buffer pH 5), 2 ml acetate buffer and 1 ml enzyme. The reaction was allowed to proceed for 30 minutes and then was terminated by addition of 1.5 ml of 1.68 N NaOH. The reaction terminated immediately (0 min.) served as control. The change in OD of pale yellow colour complex developed was read at 420 nm. The soluble proteins in the enzyme extract were estimated according to method of Lowry *et al.*, (1951) described in latter part of the chapter. The enzyme activity is expressed as change in OD $h^{-1}mg^{-1}$ protein.

**g) Enzyme alkaline Phosphatase (EC. 3.1.3.1)**

Enzyme alkaline phosphatase was assayed according to the method of Weimberg (1970). 0.5 g of germinating seed material from each treatment was homogenized separately in 8 ml of 0.1M Tris HCl buffer (pH 8.0) containing 1M KCl, 0.01M EDTA, 0.4ml 0.2M β – mercaptoethanol in chilled mortar with pestle. The resultant homogenate was filtered through two layers of cheesecloth and filtrate was centrifuged at 14,000 rpm for 20 minutes. The supernatant served as enzyme source.

The assay mixture contained 1 ml 0.1 M Tris-HCl buffer (pH 7.5), 0.1 ml of 0.05 M $MgCl_2$, 0.2 ml extract, 0.1 ml 0.02M p-nitrophenyl phosphate and 1.7ml distilled water then incubated for 60 min. The optical density of pale yellow colour developed was read at 410 nm. The soluble proteins estimated according to method of Lowry *et al.,* (1951) described in latter part of chapter. The enzyme activity expressed as change in OD $h^{-1}mg^{-1}$ protein.

**h) Enzyme Nitrate Reductase (EC.1.6.6.1)**

Activity of enzyme nitrate reductase was determined following the in vivo method described by Jaworski (1971). Germinating seeds from different treatments were taken and the seed coat was carefully removed. The seeds were cut into small pieces. A weighed amount of seed pieces incubated in the medium containing 1 ml 1M $KNO_3$, 2 ml 5% n-propanol, 5 ml, 0.2M phosphate buffer pH 7.5 and 2 ml 0.5% Triton X-100 for 1 h in dark under anaerobic conditions. After 1 hour, 1 ml of reaction mixture transferred to another test tube and mixed with 1 ml 1% sulfanilamide in 2N HCl and 1 ml 0.2% NEEDA

[N- (1-Napthyl) ethylene diamide dihydrochloride]. While mixture of 1 ml incubation medium, 1 ml sulfanilamide, 1 ml NEEDA served as blank. The absorbance was read at 540 nm on Spectronic-20. Standard curve was prepared with the help of different concentrations of $KNO_2$, and enzyme activity is expressed as μ moles of $NO_2$ liberated $h^{-1}g^{-1}$ fresh tissue.

**i) Enzyme Catalase (EC 1.11.1.6)**

Activity of enzyme catalase was studied following the method described by Luck (1974) modified by Sadasivm and Manikam (1991). 1 g of germinating seeds were homogenized in 10 ml of 1/15 m phosphate buffer pH and then filtered through four-layered cheesecloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as enzyme source.

The reaction mixture contained 3 ml of 0.03 ml $H_2O_2$ in phosphate buffer (pH 7.0) and 0.1 ml enzyme extract. Absorbance was recorded immediately at 240nm on Shimadzu 190. This was 0-min. reading. Absorbance after 3 minutes also recorded. The soluble proteins in the enzyme extract were assayed according to Lowry *et al.*, (1951) method.

Based on the change in absorbance, the enzyme activity was expressed in units $mg^{-1}$ protein.

**j) Enzyme Peroxidase (EC.1.11.1.7)**

Peroxidase activity was assayed according to method of Mahely (1954). The seed coat was removed and one g. of germinating seeds were homogenized in 10 ml of 1/15 M phosphate

buffer (pH 6.8) and filtered through 4 layered cheesecloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as enzyme source.

The reaction mixture contained 5 ml of 1/15 M acetate buffer (pH 5), 0.5 ml of 0.1% guaicol, 1 ml enzyme extract, 2 ml distilled water

0.5 ml of 0.08% $H_2O_2$ were mixed and incubated at $30^0$C for 5 minutes. After 5 minutes of incubation, 1 ml of 1N $H_2SO_4$ was added to stop the reaction. The absorbance of 0 min and 5 min reaction mixtures were at 470 nm. The soluble proteins in the enzyme extract were assayed according to method of Lowry *et al.*, (1951). The enzyme activity is expressed as change in OD per hour, $mg^{-1}$ protein.

**k) Soluble Proteins:**

The soluble proteins in enzyme extracts for various enzymes mentioned earlier were estimated by the method of Lowry *et al.*, (1951). 0.1 ml of enzyme extract was taken in test tube and diluted to 1 ml with distilled water, to this 5 ml of reagent C (50 ml of 'A' containing 2% sodium carbonate in 0.1 N aqueous NaOH in mixed with 1 ml of 'B' containing 0.5% copper sulphate in 1% Na-K tartarate), solution was mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes 0.5 ml of Folin-Ciocalteau phenol reagent was added readily with immediate mixing. This was allowed to stand for 30 minutes and intensity of developed blue color was measured at 660 nm on Spectronic-20. Protein concentration was calculated by comparing with standard curve of

different concentration of Bovine Serum albumin (0.1 mg/ml) prepared in a similar manner.

**B] PIGMENTS ANALYSIS:**

The pigment analysis was performed in The Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan, with the help of Prof. T. Hara and Prof. A. Tanaka. To study the effect of salicylic acid on pigments, seeds of wheat and moong were allowed to germinate in different concentrations of salicylic acid, in growth chambers under normal light and at $28^0$C. The seedlings of wheat and moong were harvested after 120 hr. Extraction of the pigments and analysis of the extracts by HPLC was done according to the method of Gilmore and Yamamoto (1991). 5g seedlings leaf powder was mixed with 1ml of 100% acetone and centrifuged at 144 rpm for three minutes. The supernatant was injected in HPLC for analysis of pigments. The level of various pigments was determined by comparing with the peaks of standards.

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The values of various metabolic parameters depicted in the figures in the part of result and discussion represent average of these determinations.