**Determination of Malondialdehyde Content**

Lipid peroxidation can be determined by estimating the malondialdehyde (MDA) content according to the method of Ohkawa *et al*., (1979).

Weigh 0.2g of fresh shoot and root tissues.

Homogenize the fresh tissues with liquid nitrogen by using cold mortar and pestle.

Suspend in 1 ml of 5 % Trichloroacetic acid (TCA).

Transfer homogenates into eppendorf tubes and centrifuged at 12000 rpm for 15 minutes at room temperature.

Recover supernatants into new eppendorf tubes. (Know exact volumes of supernatants!)

Add equal volume of freshly prepared 0.5% Thiobarbituric acid (TBA) in 20 % TCA onto supernatant.

Incubate the tubes for 25 minutes at 96º C by using heat block/water bath.

Keep tubes on ice after incubation until they reach the room temperature and then centrifuge them at 10000 rpm for 5 mins.

Read the absorbance of the supernatant at 532 nm (corrected for non-specific turbidity by subtracting the absorbance at 600 nm). ( Use 0.5% TBA in 20% TCA as blank)

MDA contents can be calculated using an extinction coefficient of 155 mM-1cm-1.

**Measurement of Membrane Leakage**

Membrane leakage can beestimated by measurement of electrolyte leaked from leaves and roots according to the method of Nanjo *et al*. (1999).

For conductance of leaves, 6 leaves per plant; for conductance of roots total root tissues will be put into separate 15 ml falcon tubes and immersed in 5 ml of 0.4 M mannitol at room temperature with gentle shaking for 3 h.

Measure initial conductivity electrical conductance by using Mettler Toledo MPC 227 conductivity meter (denoted C1).

Then put the tubes containing the samples into boiling water for 10 minutes.

Keep the tubes on ice until they reach to the room temperature.

Measure total conductivity by complete membrane disintegration by conductivity meter (denoted C2).

The conductivity due to leakage is expressed as the percentage of the initial conductivity over the total conductivity [(C1/C2)\* 100].

**Determination of Proline Content**

Proline amount can be determined according to the method of Bates *et al*., (1973).

Homogenize 0.2 g of shoot and root tissues with liquid nitrogen by using mortar and pestle.

Suspend the homogenate in 1 ml of 3% sulphosalicylic acid.

Transfer the extracts into eppendorf tubes and centrifuge at 14000 rpm for 5 minutes at 4ºC.

Prepare an eppendorf tube containing 0.2 ml acid ninhydrin (0.31 g ninhydrin, 7.5 ml acetic acid and 5 ml 6M phosphoric acid), 0.2 ml 96% acetic acid and 0.1 ml 3% sulphosalicylic acid for each sample.

After centrifugation, add 0.1 ml of supernatant from each extract into the eppendorf tubes. Immediately mix the tubes gently by inverting.

Incubate the tubes at 96ºC for 1 hour by using heat block or water bath (for the hydrolysis of proteins).

After incubation, add 1 ml toluene and vortex the tubes.

Centrifuge the tubes at 14000 rpm for 5 minutes.

Transfer, the pink-red colored upper phase into cuvettes and read absorbance at 520 nm (use toluene as blank).

To estimate the the proline concentration, prepare a standard curve between the range of 5μm-500μm.

**Measurement of H2O2 Content**

H202 content can be estimated according to the method of Bernt and Bergmeyer (1974), using peroxidase enzyme.

Grind 0.5 g shoot and root tissues using a cold mortar and pestle with liquid nitrogen.

Suspend the powders in 1.5 ml of 100 mM potassium phosphate buffer at pH 6.8.

Transfer the homogenates into centrifuge tubes and centrifuge at 18000 g for 20 min at 4ºC.

To start the enzyme reaction, put 0.25 ml supernatant and 1.25 ml peroxidase reagent consisting of 83 mM potassium phosphate buffer at pH 7.0, 0.005% (w/v) o-dianizidine, 40 μg peroxidase/ml, in an eppendorf tube and incubate for 10 min at 30ºC in a waterbath.

Stop the reaction by adding 0.25 ml of 1N perchloric acid.

Centrifuge the tubes at 5000 g for 5 minutes.

Read the absorbance of the supernatant at 436 nm.

Determine the amount of hydrogen peroxide by using an extinction coefficient of 39.4 mM-1cm-1.

**Protein Determination**

The protein amounts in the root and shoot crude extracts can determined by Bradford method (Bradford, 1976).

In order to prepare the Bradford reagent, dissolve 500 mg of Coomassie Brillant Blue G-250 in 250 ml of 95% ethanol. Add 500 ml of 85% phosphoric acid to the mixture and the solution, then dilute the solution to 1L with dH20 and filter. (This will be 5X stock solution)

Dilute 5X stock solution to 1X before each use.

Take 20μl from shoot and 40μl from root samples and dilute them to 500μl with dH20. Add 5 ml of 1X Bradford reagent to the diluted sample.

Incubate the tubes 10 minutes at room temperature.

Then, read the absorbance at 595 nm by using spectrophotometer against a blank of 500 μl water and 5 ml 1X Bradford reagent.

Prepare a standard curve by using Bovine Serum Albumin (BSA) with concentrations of 10, 20, 30, 40, 50, 60 μg/ml.

**Determination of APX Activity**

Ascorbate peroxidase activity can be determined according to the spectrophotometric method of Wang *et al*., (1991).

Grind 0.5 g of shoot and root tissues with liquid nitrogen and suspend the powders in 1 ml of suspension solution containing 50 mM Tris-HCl (pH 7,2), 2% PVP, 1 mM EDTA and 2mM ascorbate.

Transfer the homogenate into eppendorf tubes and centrifuge them at 12100 g for 20 minutes at 4ºC.

Recover the supernatants for enzyme assay.

Add enzyme extract containing 100 μg protein, determined by Bradford method, into the assay medium consisting of 50 mM potassium phosphate buffer (pH 6.6) , 0,25 mM ascorbate and 1mM H2O2. (Addition of hydrogen peroxide initiates the reaction.)

Record the decrease in the ascorbate concentration at 290 nm with spectrophotometer for 1.5 minutes. Assay medium without enzyme extract is used as blank.

The enzyme activity is calculated from the initial rate of the enzyme. (Extinction coefficient of ascorbate = 2.8 mM-1cm-1 at 290 nm).

Ascorbate + Hydrogen peroxide → Dehydroascorbate + Water

C6H8O6 + H2O2 → C6H6O6 + 2 H2O

**Determination of CAT Activity**

Activity of catalase can be determined according to the method of Chance *et al*. (1955).

0.5 g of tissue from shoots and roots will be ground in a cold mortar and pestle and then suspended in 1 ml of 50 mM Tris-HCl suspension solution at pH 7.8.

Centrifuge the homogenates at 12000 g for 20 min at 4ºC. Use the supernatants for the enzyme assay. Enzyme extract containing 50 μg protein (determined by Bradford method) will be added into assay medium containing 50 mM potassium phosphate buffer at pH 7 and 10 mM H2O2. By the additon of the enzyme extract the reaction starts.

Record the decrease of the H2O2 absorbance at 240 nm with Schimadzu spectrophotometer for 1.5 minutes.

The enzyme activity is calculated from the initial rate of the enzyme. (Extinction coefficient of H2O2 = 39,4 mM-1cm-1.)

Hydrogen peroxide (H2O2) → Water (H2O) + Oxygen (O2)

**Determination of GR Activity**

Glutathion reductase activity can be determined according to the method of Sgherri *et al.,* (1994).

Homogenize 0.5 g of tissue with liquid nitrogen by using cold mortar and pestle.

Suspend the powder in 0,75 ml of suspension solution containing 100 mM potassium phosphate buffer (pH 7.0) , 1 mM Na2EDTA and 2% insoluble PVP.

Centrifuge the suspensions at 18000 g for 20 min at 4ºC and the supernatants are used for the enzyme assay.

Enzyme extract containing 75μg protein (determined by Bradford method) will be added into assay medium containing 200 mM potassium phosphate buffer (pH 7.5), 0,2 mM Na2EDTA, 1,5 mM MgCl2, 0,50 mM GSSG, 50 μM NADPH, in a final volume of 1 ml.

By the addition of NADPH the reaction starts.

Assay medium without enzyme extract is used as blank.

The decrease in the NADPH concentration will be recorded at 340 nm for 1.5 minutes.

The enzyme activity is calculated from the initial rate of the enzyme. (Extinction coefficient of NADPH = 6.2 mM-1cm-1 at 340 nm)

Glutathione disulfide (GSSG) + NADPH → Glutathione (2GSH) + NADP+

**Determination of SOD Isozyme Activities**

One dimensional native polyacrylamide gel electrophoresis is used to estimate the SOD activity and SOD isozyme patterns. Gels are stained by negative activity staining technique according to the method of Beuchamp and Fridovich (1971).

Superoxide (2O2.-) + 2H**+** → Hydrogen peroxide (H2O2) + Oxygen (O2)

**Preparation of Shoot Crude Extracts**

0,2 g of shoot tissues are weighed and homogenized by using cold mortar and pestle with liquid nitrogen.

The powder is suspended in 800 μl of homogenization buffer consisting of 9 mM Tris-HCl at pH 6.8 and 13.6% glycerol.

The susupensions are then transferred into eppendorf tubes and centrifuged at 14000 rpm for 5 minutes by using Hettich microcentrifuge.

Supernatants are used for the SOD assay.

**Preparation of Root Crude Extracts**

0,5 g of root tissues are weighed and homogenized by using cold mortar and pestle with liquid nitrogen.

The powder is suspended in 750 μl of grinding buffer consisting of 0.2 M sodium phosphate buffer and 2 mM EDTA at pH 7.5.

The suspensions are then centrifuged at 10000 g for 30 minutes at 4ºC and supernatants are used for the SOD activity assay.

**One-Dimensional Native Polyacrylamide Gel Electrophoresis (1-D PAGE)**

Seperating gel (5 ml 12%) and stacking gel (2,5 ml 5%) are prepared to carry-out 1-D PAGE according to the method of Laemmli (1970).

Gels are polymerized in Cleaver Minigel Apparatus. Equal amounts of proteins determined by Bradford method (30 μg/ well) are loaded to the wells.

5μl standard SOD protein is also applied in one of the wells to determine the intensities. (0,1 unit/ μl) Electrophoresis is carried out under constant current of 6 mA in stacking gel and 10 mA in seperating gel for approximately 4 hours.

**Negative Activity Staining**

Gels are transferred into glass containers including 25 ml 50 mM potassium phosphate buffer at pH 7.5, 0,1 mM EDTA, 0,2 % (v/v) N,N,N’N’- tetramethyl ethylene diamine (TEMED), 3mM riboflavin and 0,25 mM nitroblue tetrazolium.

After a dark incubation period for 45 minutes with occasional shaking, gels are washed with dH2O several times under illumination, until the colour development occurs.

The changes in SOD isozymes are evaluated from band intensities.

The unit SOD activity is determined by running a SOD Standard from bovine erythrocyte. One unit of Standard SOD activity inhibits the rate of reduction of Cyt c by 50% in coupled system with xanthine oxidase at ph 7.8 at 25ºC in a 3 ml reaction medium.

**SOD Isozyme Determination**

At the end of electrophoresis, the gel is cut into three.

Two of them are incubated for 15 minutes in seperate inhibition solutions; one containing 50 mM potassium phosphate buffer (pH 7.8), 0,5 mM EDTA and 5 mM KCN, and the other containing 5 mM H2O2.

The other gel is incubated in 50 mM potassium phosphate buffer (pH 7.8) and 0,5 mM EDTA as control.

Then the gels are treated with negative activity stain as described above.

MnSOD is resistant to both H2O2 and KCN, FeSOD is resistant only to KCN and Cu/ZnSOD is sensitive to both inhibition solutions.