APPENDIXES

APPENDIX-I

Estimation of Catalase Activity

(Luck, 1974)

Principle

The UV light absorption of hydrogen peroxide solution can be measured between 230 and 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease. But this method is applicable only to enzyme solution, which do not absorb strongly at 230 - 250 nm.

Reagents

1. Phosphate buffer 0.067 M (pH 7.0): Dissolved 3.522 g of KH_2PO_4 and 7.268 g of $K_2HPO_4.2H_2O$ in distilled water and made up the volume to 1 litre.

2. Hydrogen peroxide - phosphate buffer: Dissolved 0.16 ml of H_2O_2 (10% w/v) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240 nm with 1 cm light path.

Procedure

Enzyme extract:

The sample homogenized in a prechilled mortar and pestle with 0.067M phosphate buffer at 1-4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24hours.The combined supernatants were used for the assay.

Assay

Pipetted out 3.0 ml of H_2O_2 Phosphate buffer into the experimental cuvette. Mixed with 0.01 - 0.04ml of sample with a glass rod flattened at one end. Noted the time Δt required for a decrease in absorbance from 0.45 to 0.4 at 240 nm. This value was used for the calculation. If Δt was greater than 60 seconds, then repeated the measurements with more concentrated solution of the sample. Calculated the activity and expressed in units per mg protein. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

Calculation

Calculated the concentration of H_2O_2 using the extinction coefficient 0.036 μ mole/ml.

APPENDIX -II

Estimation of Peroxidase Activity

(Reddy *et al.*, 1995)

Principle

Peroxidase converts H_2O_2 to water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine) the oxidation of pyrogallol or dianisidine to colored product called purpurogalli is measured calorimetrically.

$$\text{Pyrogallol} + H_2O_2 \rightarrow \text{Oxidized pyrogallol} + H_2O_2$$

Reagents

1. Pyrogallol – 0.05 phosphate buffer (pH 6.5)
2. 1% H_2O_2

Enzyme extract

Measured one gram of the sample with 5ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuge the homogenate at 300g for 15minutes. Used the supernatant as the enzyme source. All procedures were carried out at 0-50° C.

Procedure

Pipetted out 3ml of 0.05 M- Pyrogallol solution and 0.5 to 0.1ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400 nm. Added 0.5ml of 1% H_2O_2 in the cuvette. Recorded the change in the absorbance every 30 seconds up to 3 minutes.

Calculations

Change in absorbance/min = X

Weight of the plant material taken = 300mg

Volume of extract taken for the assay = 0.02ml

Change in absorbance = X

Change in absorbance 1.5ml extract = (X/0.02) x 1.50-Y

(i.e.) Peroxidase activity in 300mg plant tissue = Y

Peroxidase activity/gram of Plant tissue = Y x (1000/300) Units.

APPENDIX-III

Assay of Superoxide Dismutase

(Misra and Fridovich, 1972)

Principle

Superoxide dismutase (SOD) uses the phytochemical reduction of riboflavin as oxygen generating system and catalyzes the inhibition of Nitro blue tetrazolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600 nm.

Reagents

1. 50mM Potassium phosphate buffer
2. 45μM Methionine
3. 5.3μM Riboflavin
4. 84μM NBT
5. 20mM Potassium cyanide

Procedure

The incubation medium contained 300μl of each reagent (50mM potassium phosphate buffer, 45μM Methionine, 5.3μM Riboflavin, 84μM NBT and 20mM potassium cyanide). To the test 300μl of sample was added and the final volume was made up to 3 ml with distilled water. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The maximum reaction was evaluated in the absence of enzyme. One unit of enzyme activity is defined as the enzyme reaction, which gives 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units.

APPENDIX -IV

Estimation of Polyphenol Oxidase Activity

(Ester Bauer *et al.*, 1977)

Principle

Polyphenol oxidases are copper proteins of wide occurrence in nature, which catalyses the aerobic oxidation of certain phenolic substrate to quinine, which are auto oxidized to dark pigments generally known as melanin. The polyphenol oxidases (PPO) comprise catechol oxidase and laccase. One unit of either catechol oxidase or laccase is defined as the amount of enzymes that transforms 1µmol of quinine per minute under the assay conditions.

Reagents

1.50Mm Tris-HCL (pH 7.2)

2. 0.4M sorbitol

3. 10Mm sodium chloride

4. 0.1M Phosphate buffer

5. 0.01M catechol solution

Preparation of enzyme extract

Ground about 5g of the tissue and made up to 20ml with the medium containing 50mM Tris – HCL (pH 7.2), 0.4M Sorbitol and 10Mm NaCl. Centrifuged the homogenate at 2000rpm for 10 min and used the supernatant for the assay.

Procedure

Added 2.5ml of 0.2M phosphate buffer (pH 6.5), 0.3ml of catechol solution (0.01M) into the cuvette and set the spectrophotometer at 495nm.

Now added the enzyme extract (0.2ml) and started recording the change in absorbance for every 30 seconds up to 5 minutes.

Enzyme units in the test = K*(^/min)

K for catechol oxidase = 0.272

K for laccase = 0.242

APPENDIX-V

Estimation of Glutathione-S-Transferase Activity

(Habig *et al.*, 1974)

Principle

The enzyme activity is measured by its ability to conjugate glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB), the extent of conjugation causing a proportionate change in absorption at 340nm.

Reagents

1. 1μM CDNB in ethanol.
2. 1μM Glutathione (GSH).
3. 0.1M Phosphate buffer.
4. 50μM Tris-HCl buffer pH (7.2).

Procedure

Enzyme extract

The sample was homogenized with Tris-HCl buffer (pH 7.2). The homogenate was centrifuged at 4°C for 30 minutes at 8500rpm. The supernatant was used as the enzyme source.

Assay

The assay was done at 25°C under conditions giving activities linear with respect to incubation times and protein concentrations for atleast 3 minutes.

The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. 0.1ml of both substrates (GSH and CDNB) was taken in 0.1M phosphate buffer pH 6.5 at room temperature to make a volume of 2.9ml. The experiment was started by adding 0.1ml of sample to this mixture and the readings were recorded against distilled water blank for a minimum for 3 minutes. The complete assay mixture without the sample served as the control to monitor non-specific binding of substrates. Care was taken to ensure that the final concentration of ethanol in mixture was always less than 4 percent.

Calculation

GST activity was calculated using the extinction co-efficient of the product formed and the values have expressed as nanomoles of CDNB conjugated per minute per gram sample.

$$\text{Activity} = \frac{\text{OD}\times 3\times 1000}{9.6\times 5\times \text{protein in mg}}$$

APPENDIX-VI

Assay of Glutathione Peroxidase Activity

(Rotruck *et al.*, 1973)

Principle

A known amount of enzyme preparation was used to react with H_2O_2 in the presence of GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman.

$$2GSH + H_2O_2 \xrightarrow{Se-GPx} GSSG + 2\ H_2O_2$$

Reagents

1. 0.4M Tris buffer
2. 10µM Sodium azide
3. 10% TCA
4. 0.4µM EDTA
5. 10µM Hydrogen peroxide
6. 2µM Glutathione

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of plant extract were added. 0.2ml of glutathione followed by 0.1ml of hydrogen peroxide were added to the mixture, mixed well and incubate at 37°C for 10 minutes along with a tube containing all the reagents expect sample. After 10 minutes the reaction was arrested by the addition of 0.5ml of 10% TCA and centrifuged and the supernatant was assayed for glutathione by the method of Ellman.

The activities are expressed as µg GSH consumed / minute /mg protein.

APPENDIX-VII

Estimation of Glutathione Reductase Activity

(David and Richard, 1983)

Principle

Glutathione reductase catalyses the conversion of oxidized glutathione to reduced glutathione by employing NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

Reagents

1. 0.12M Phosphate buffer
2. 15μM EDTA
3. 10 μM Sodium azide
4. 6.3μM Oxidized glutathione
5. 9.6μM NADPH

Procedure

1g of sample was homogenized with 0.12M phosphate buffer centrifuged at 500rpm for 10 minutes and supernatant was used as the source of enzyme.

Assay

The assay system contained 1ml of 0.12M potassium phosphate buffer, 0.1ml EDTA, 0.1ml of 10μM Sodium azide, 0.1ml of 0.3μM oxidized glutathione and 0.1ml of enzyme source and made upto 2.0ml with water. Kept for 3 minutes at room temperature. Then 0.1ml of NADPH was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 2-3 minutes. Control was carried out which contain water instead of oxidized glutathione. The enzyme activity was expressed as μmoles of NADPH oxidized / minute / gram sample.

APPENDIX-VIII

Estimation of Ascorbic acid

(Roe and Keuther, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbic acid then reacts with 2, 4-dinitrophenylhydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotometrically at 540nm.

Reagents

1. 4% Trichloro acetic acid
2. 9N Sulphuric acid
3. 2% 2, 4-dinitrophenylhydrazine reagent (DNPH)
   Dissolved 2g of DNPH in100ml of 9N sulphuric acid
4. 10% Thiourea
5. 85% Sulphuric acid
6. Stock standard solution
   Dissolved 100mg of ascorbic acid in 100ml of 4% TCA
7. Working standard solution
   Diluted 10ml of stock solution to 100ml with 4% TCA

Procedure

Ground 1g of the sample and homogenized in 4% TCA. Made upto 10ml and centrifuged at 2000rpm for 10 minutes. The supernatant obtained was treated with a pinch of activated charcoal, shaken well and kept for 10 minutes.

Centrifuged once again to remove the charcoal residue. Noted the volume of clear supernatant obtained.

0.5 and 1ml aliquots of this supernatant were taken for the assay. The assay volume made upto 2.0ml with 4% TCA. 0.2 to 1.0ml of working standard solution containing 20 - 100µg of ascorbate respectively was pipetted out into clean dry test tubes and the volume was made upto 2ml with 4% TCA.

Added 0.5ml of DNPH reagent to all the test tubes followed by two drops of 10% thiourea solution. Incubated at 37°C for 3 hours. The osazone formed was dissolved in 2.5ml of 85% sulphuric acid, in cold, drop by drop with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30minutes at room temperature, the absorbance was read spectrophotometrically at 540nm.

Calculated the content of ascorbic acid in the plant sample using the standard graph.

APPENDIX-IX

Estimation of α-Tocopherol

(Emmeric-Engel method 1938 as described by Rosenberg, 1992)

Principle

Tocopherol can be estimated using Emmeric-Engel reaction which is based on the reduction of ferric to ferrous ions by tocopherol, which then forms a red colour with 2, 2'dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction is read at 460nm to measure carotenes. Correlation is made for the carotenes after adding ferric chloride and reading at 540nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2, 2'-dipyridyl

   1.2g in one litre of n-propanol
4. Ferric chloride solution

   1.2g of Fe $Cl_3.6H_2O$ in one litre ethanol. Stored in a brown bottle.
5. Standard solution of D,L –α-tocopherol

   100mg/100ml of α-tocopherol in absolute alcohol. 91mg of α-tocopherol equivalent to 100mg of tocopherol acetate.

Extraction of plant tissue

The sample was homogenized in a blender. Weighed accurately 2.5g of the homogenized tissue into a conical flask. Added 50ml of 0.1N sulphuric acid slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatmann No.1 filter paper discarding the initial 10 -15 ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure

Into three stoppered centrifuge tubes (test, standard and blank) pipette out 1.5ml of sample extract, 1.5ml of standard and 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard, added 1.5ml of water. Added 1.5ml of xylene to all the tubes stoppered, mixed well and centrifuged.

Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any ethanol or protein. Added 2ml of 2, 2'-dipyridyl reagent

to each tube stoppered and mixed. Pipetted out 1.5ml of the mixture into the spectrophotometer cuvette and read the extinction of the test and the standard against the blank at 460nm. Then in turn, beginning with the blank, added 0.33ml of ferric chloride solution. Mixed well and after exactly 15 minutes read test and standard against the blank at 520nm. The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherol in } \mu g = \frac{Reading\ at\ 520 - Reading\ at 460nm}{Reading\ of\ standard\ at\ 520nm} \times 0.24 \times 15.$$

APPENDIX-X

Estimation of Reduced Glutathione

(Moron *et al.*, 1979)

Principle

Reduced glutathione is measured by its reaction with 5, 5'-dithio 2-nitro benzoic acid (DTNB) to give a compound that absorbs at 412nm.

Reagents

1. 5% TCA
2. Sodium phosphate buffer
3. DTNB solution

   0.6μM DTNB in 0.2M phosphate buffer
4. Standard glutathione

   Dissolved 10mg of reduced glutathione in 100ml of 5% TCA

Procedure

1g sample was homogenized in 5% TCA to give a 20% homogenate. The precipitated protein was centrifuged down at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of supernatant was taken for estimation. The volume of the aliquot was made upto 1ml with 0.2M sodium phosphate buffer (pH 8.0). 2ml of freshly prepared DTNB solution was added to all the tubes and the intensity of yellow colour formed was read at 412nm in a spectrophotometer after 10minutes.

A standard curve of GSH was prepared using concentrations ranging from 2 to 10 nanomoles of GSH in 5%TCA.

APPENDIX- XI

Estimation of Polyphenol

(Malick and Singh, 1980)

Phenols, the aromatic compounds with hydroxyl groups are wide spread in plant kingdom. They occur in all parts of the plants. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amounts of polyphenols are resistant to bird attack. Phenols include an array of compounds like tannins, flavonols etc., Total phenol estimation can be carried out with Folin-Ciocalteau reagent.

Principle

Phenol react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrophotometer at 650nm.

Reagents

1. 80% Ethanol

2. Diluted Folin-ciocalteau reagent

3. 20% sodium carbonate

4. Stock solution: Dissolved 100mg of catechol is made up to with 100ml distilled water.

5. Working standard: 10ml of stock standard was diluted to 100ml.1.0ml of this contains 100µg of catechol.

Procedure

1g of sample was homogenized using 20ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes .The supernatant was saved. The residue was reextracted with 10ml of 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50ml) and 2.0ml was taken for the experiment. A working standard of 0.5-2.5ml catechol solution corresponding to 50-250µg of catechol were pipetted out into a series of test tubes. The volume was made up to 2.5ml with water. To all the tubes added 0.5ml of diluted Folin-ciocalteau reagent. After 3 minutes, added 2.0ml of 20%Na_2CO_3 solution to each test tube and mixed thoroughly. The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank.

APPENDIX-XII

Estimation of Total Carotenoids and Lycopene

(Zakaria *et al.*, 1979)

Principle

The total Carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in UV/visible spectrophotometer at 450nm.

Lycopene has the absorption maximum at 473nm and 503nm. A rapid method for the estimation of Lycopene in plant product is based on the movement of absorption of the petroleum ether extract of the total carotenoids at 503nm. After measuring the total carotenoids at 450nm, the same extract can be used for estimating Lycopene at 503nm. At 503nm Lycopene has a large absorption while carotenoids have only negligible absorbance.

Reagents

1. Petroleum ether
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. 12% alcoholic KOH (ice cold)

Procedure

Weighed 5-10g of the sample. Saponified for about 30minutes in a shaking water both at 37°C after extracting the sample in 12% alcoholic KOH. Transferred the saponified extract into a separating funnel (packed with glass wool and calcium carbonate) containing 10-15ml of petroleum ether and mixed gently. Taken up the carotenoid pigments into the petroleum ether layer. Transformed the lower aqueous phase into another separating funnel and the

petroleum ether extract containing carotenoid pigments to an amber-coloured bottle. Repeated the extraction of the aqueous phase similarly with petroleum ether, until it became colourless. Discarded the aqueous phase. To the petroleum ether extract added a small quantity of anhydrous sodium sulphate to remove turbidity. Noted the final volume of petroleum ether extract and diluted if needed by a known dilution factor.

The absorbance of the extract at 450nm and 503nm was noted in a spectrophotometer using petroleum ether as a blank.

For total carotenoids

$$\text{Amount of total carotenoids present} = \frac{P \times 4 \times V \times 100}{W} \mu g$$

Where, P – Optical density of the sample

V – Volume of the sample

W – Weight of the sample

For Lycopene

Lycopene

$$(\text{mg}/100\text{g sample}) = \frac{3.1206 \times \text{OD of the sample} \times \text{Volume made up} \times \text{dilution } 100}{1 \times \text{Weight of the sample} \times 1000}$$