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# Appendices

## Appendix 1

### Estimation of total chlorophyll

(Yoshida *et al.*, 1971)

#### Spectrophotometric Method

Chlorophyll pigments present in the sample are extracted using acetone (80%) and the green colour of the extract is read in a spectrophotometer at 645 nm and at 663 nm and the total chlorophyll was calculated using the formula

$$\text{Total chlorophyll} = \frac{20.2 A_{645} + 8.02 A_{663}}{a \times 1000 \times w} \times V \text{ mg g}^{-1}$$

where, a = path length of the light in the cell (usually 1 cm), v = volume of the extract in ml, w = fresh weight of the sample in g.

## Appendix 2

### Estimation of soluble protein

(Lowry *et al.*, 1951)

#### Spectrophotometric Method

The method is based on the principle that different proteins contain different amounts of aromatic residues which react with Folin-Ciocalteu reagent giving a blue colour which is read in a colorimeter at 750 nm ( Alkaline copper reagent: Reagent A – 2% sodium carbonate in 0.1 N sodium hydroxide, reagent B – 0.5% copper sulphate in 1% sodium potassium tartarate. Mixed 50 ml reagent A and 1.0 ml of reagent B freshly before use, 1N sodium hydroxide, Folin-Ciocalteu reagent, Protein standard solution: 50 mg / 100ml of bovine serum albumin was made up with 0.9% saline.

#### Procedure

Into a series of test tubes pipetted out 0.2 to 1.0 ml of standard albumin solution. Made up to 1.0 ml with distilled water. To 1.0 g of the sample added 1.0 ml of 1N sodium hydroxide and heated to 100 ° C for five minutes. Extracted 1.0 ml of this solution for protein estimation. To the test and standard tubes added 5.0 ml of alkaline copper reagent and allowed the mixture to stand at room temperature for ten minutes. Added 0.5 ml of Folin-Ciocalteu reagent rapidly and mixed immediately. After thirty minutes measured the absorbance at 750 nm in a colorimeter. From the standard graph, calculated the amount of protein present in the sample.

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### Appendix 3

#### Estimation of proline

(Bates *et al.*, 1973)

#### Spectrophotometric Method

During selective extraction with aqueous sulphosalicylic acid, proteins are precipitated as a complex. Other interfering materials are also presumably removed by absorption to the protein sulphosalicylic acid complex. The extracted proline is made to react with ninhydrin in acidic conditions (pH 1.0) to form the chromophore (red colour) and read at 520 nm using the reagents namely acid ninhydrin, 3% aqueous sulphosalicylic acid, glacial acetic acid, toluene and proline.

#### Procedure

Extracted 0.5 g of plant material by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. Filtered the homogenate through Whatman No.2 filter paper and took 2 ml of filtrate in a test tube and added 2 ml of glacial acetic acid and 2 ml of acid ninhydrin. Heated in a water bath for 1 hr. Terminated the reaction by placing the tube in ice bath. Added 4 ml toluene to the reaction mixture and stirred well for 20-30 seconds. Separated the toluene layer and warmed to room temperature. Measured the red colour intensity at 520 nm. Standard proline was run in a similar way and standard curve was prepared. From the standard, the amount of proline in the plant sample was calculated.

### Appendix 4

#### Estimation of oil in oilseeds

(Cox and Pearson, 1962)

#### Soxhlet Extraction Method

Oil from a known quantity of the seed is extracted with petroleum ether. It is then distilled off completely, dried, the oil weighed and the percentage of oil is calculated. Folded a piece of filter paper in such a way to hold the seed meal. Wrapped around a second filter paper which was left open at the top like a thimble. A piece of cotton wool was placed at the top to evenly distribute the solvent as it drops on the sample during extraction. Placed the sample packet in the butt tubes of the Soxhlet extraction apparatus and extracted with petroleum ether (150 drops / min) for 6 hr without interruption by gentle heating. Allowed to cool and dismantled the extraction flask. Evaporated the ether on a steam or water bath until no odour of ether remained. Cooled at room temperature. Carefully removed the dirt or moisture outside the flask and weighed the flask. Repeated heating until constant weight was recorded. The percentage of oil was calculated using the formula - Oil in ground sample % =  $\frac{\text{Weight of oil (g)}}{\text{Weight of sample (g)}} \times 100$

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**Appendix 5**  
**Fatty acid profile of groundnut oil**  
**High Pressure Thin Layer Chromatography Method**

High Pressure Thin Layer Chromatography was run to compare the fatty acid profile of the different varieties of groundnut. 100 µl of oil sample was dissolved in 900 µl diethyl ether, which was used as the test solution for analysis. 2µl of each test solution was loaded as 5mm band length in the 15 x 10 Silica gel 60F<sub>254</sub> TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturating with solvent vapor) with the N-hexane-diethyl ether-glacial acetic acid (8: 2: 0.1) mobile phase, and the plate was developed up to 80mm.

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254nm and UV 366nm. The developed plate was sprayed with 0.05% ethanolic rhodamine spray reagent and dried at 100° C in hot air oven. The plate was photo-documented in daylight and UV 366nm mode using Photo-documentation chamber. After derivatization, the plate was fixed in scanner stage and scanning was done at 366 nm. The Peak table, Peak display and Peak densitogram were noted.

**Appendix 6**  
**Determination of soil texture**  
**(Mani et al., 2007)**  
**International Pipette Method**

This method is based on Stoke's law. According to this law the rate of fall of a particle in liquid is directly proportional to the square of its radius.  $V \propto r^2$

$$V = \frac{2}{9} \times \frac{(d_p - d) \times g \times r^2}{\eta}$$

where, V=sedimentation velocity (cm sec<sup>-1</sup>), g=acceleration due to gravity (cm sec<sup>-1</sup>), r=radius of the particle or sphere (cm), d<sub>p</sub>=density of the particle (g cc<sup>-1</sup>), d=density of the liquid (g cc<sup>-1</sup>), η= viscosity of the liquid. The soil is first dispersed by destroying the binding agents with hydrogen peroxide and hydrochloric acid followed by treatment with a dispersing agent. Clay and slit are separated by sedimentation and coarse and fine sand by sieving.

**Procedure**

Transferred 20g of air dried soil sample to a 500 ml beaker. Added 60 ml of 6% hydrogen peroxide. Stirred it well and kept it on a water bath for thirty minutes till

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frothing ceases. Treated with hydrogen peroxide to destroy the organic matter. Then added 200 ml of 0.2 N hydrochloric acid, stirred it well and kept it over night. Hydrochloric acid was added to destroy calcium carbonate which is also a binding agent. Filtered the contents through Whatman No.50 filter paper and washed it with water till the filtrate was free of chloride. Formation of curdy white precipitate indicate the presence of chloride. When the filtrate was free of chloride, transferred the soil material from the filter paper to another 500 ml beaker and added about 400 ml water. Then added 8 ml of normal sodium hydroxide and stirred well for 10 minutes with a mechanical stirrer. Transferred the contents to a 1000 ml spout less measuring cylinder and made up to 1litre. Covered the cylinder tightly with a rubber stopper and shook the contents thoroughly by repeated inversions.

### **Clay and silt**

Removed the rubber stopper and placed the cylinder under Robinson pipette and started a stop clock simultaneously. Noted down the temperature and the setting time for clay plus silt from the table. Till the setting time was over the suspension was not disturbed. First lowered the pipette in such a way that the tip of the pipette just touched the surface of the suspension. At the end of the stipulated settling time for clay and silt, lowered the pipette to 10cm depth and drew 20 ml suspension and delivered it to a weighed clean porcelain dish. This suspension contained clay plus silt. Evaporated this on a water bath and dried it in an air oven at 105 °C. Cooled it in a desiccator and determined the weight of clay plus silt.

### **Clay alone**

Shook the contents of the cylinder well and allowed undisturbed till the stipulated setting time for clay alone corresponding to the suspension temperature. Withdrew 20 ml of the suspension at the end of the period as done in the case of clay plus silt and determined the weight as clay alone after evaporation and drying.

### **Coarse sand and fine sand**

Poured out major portion of the suspension from the cylinder after withdrawing sample for clay alone. While pouring out care was taken to see that no sand fraction was lost. Then washed the sediment with water and transfered the contents to a tall form beaker. Added water to a height of more than 10 cm. Stirred well and allowed it to stand for 4 minutes. Then poured off the supernatant liquid. Repeated this process till the water poured off was no longer turbid. Transferred the residue to a porcelain basin, dried it in an oven and weighed as coarse sand plus fine sand. Sieved the coarse sand and fine sand in a seventy mesh sieve. The material passing through the sieve was fine sand while the coarse sand fraction was retained on the sieve. Weighed it as coarse sand.

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**Appendix 7**  
**Estimation of available nitrogen in soils**  
**(Subbiah and Asija, 1956)**  
**Alkaline KMnO<sub>4</sub> Method**

The amount of soil nitrogen released by the potassium permanganate oxidation of part of soil organic matter is estimated by distillation with sodium hydroxide. The distillate is collected in boric acid containing double (mixed) indicator and titrated against standard sulphuric acid. The reagents used were 0.32% potassium permanganate, 2.5% sodium hydroxide, double (mixed) indicator: (Bromocresol green + Methyl red), standard sulphuric acid (N/50) and boric acid 2%.

**Procedure**

Weighed 20 g of soil and transferred to a distillation flask. Added 20 ml of distilled water and 1 ml of liquid paraffin. Added a few glass beads (with holes) (to prevent bumping) and then added 100 ml of 0.32% potassium permanganate solution and 100 ml of 2.5% sodium hydroxide solution. Distilled the contents at a steady rate collecting the liberated ammonia in a 500 ml ice tumbler containing 20 ml of boric acid with double (mixed) indicator.

**Appendix 8**  
**Estimation of available phosphorus in soils**  
**(Olsen *et al.*, 1954)**  
**Colorimetric Method**

This method of extraction of available soil phosphorus is suited for calcareous and alkaline soils. The carbonate ions from NaHCO<sub>3</sub> will react with Ca<sup>2+</sup> and CaCO<sub>3</sub> is precipitated, thus allowing the phosphorus to come into solution. The amount of phosphorus extracted was determined colorimetrically using 0.5 M sodium bicarbonate (pH 8.5), activated carbon, reagent A: ammonium molybdate, antimony potassium tartarate in 5 N sulphuric acid, Reagent B: ascorbic acid in reagent A (fresh).

**Procedure**

Weighed 5 g of soil and transferred to a 100 ml polythene shaking bottle (Run a blank side by side). Added a pinch of activated carbon (to make the extracted solution colourless). Added 50 ml of 0.5 M sodium bicarbonate and shook in a reciprocating mechanical shaker for thirty minutes. Filtered through Whatman No.40 dry filter paper, collecting the filtrate in a clean dry beaker (the filtrate should be clear and colourless). Pipetted out 5 ml of the filtrate into a 25 ml volumetric flask. Diluted the solution in the 25 ml volumetric flask to about 20 ml with distilled water. Added 4 ml of reagent B and made up the volume to 25 ml. Waited for ten minutes for the colour to develop and thereafter measured the intensity of the blue colour in a photoelectric

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colorimeter using a red filter(660 nm wavelength) and adjusting the meter to hundred percent transmittance with the blank. From the standard curve for phosphorus, determined the concentration of phosphorus (ppm) in the solution against the percent transmittance observed in the above step.

## **Appendix 9**

### **Estimation of available potassium in soils**

**(Stanford and English, 1949)**

#### **Flame Photometric Method**

The K<sup>+</sup> ions in the exchange sites are replaced with NH<sup>4+</sup> ions and leached from the soil. The K<sup>+</sup> ions in solution are then determined using the flame photometer using the reagents namely neutral normal ammonium acetate and standard potassium solution.

#### **Procedure**

Weighed 5.0 g of soil and transferred it to a 100 ml polythene shaking bottle. Added 25 ml of neutral normal ammonium acetate and shook in a mechanical reciprocating shaker for five minutes. Filtered through dry Whatman No.40 filter paper collecting the filtrate in a dry test tube or beaker or injection vial. Measured the amount of potassium in the filtrate in the flame photometer.

## **Appendix 10**

### **Estimation of indole acetic acid oxidase activity**

**(Parthasarathy *et al.*, 1970)**

#### **Colorimetric Method**

The IAAO activity is determined by measuring residual IAA following dark incubation with shaking at 30°C. The IAA is determined by Salkowski reaction. Since monophenols act as cofactors of IAAO, and o-and p-dihydroxy phenols and polyphenols act as inhibitors of this enzyme, the monophenolic compound, para coumaric acid is added in the enzyme assay for activation. The reagents used were 0.071 M phosphate buffer, para – coumaric acid solution, IAA solution, manganese chloride solution, 5 M perchloric acid, 0.1 M ferric nitrate, Enzyme extract: Prepared acetone powder from the frozen tissue by blender homogenizing 25 g tissue in two successive 100 ml aliquots of cold acetone. The homogenate was collected by Buchner filtration through Whatman No.1 filter paper following both grindings. The homogenate was air dried until free of acetone odour and the resulting dry powder was weighed and freezer stored in cold containers. One gram of acetone powder was ground in two successive 20 ml aliquots of 25 mM phosphate buffer in a mortar chilled in an ice bath. Collected the extract by Buchner filtration through Whatman No.1 paper after each grinding. Combined the filtrates and diluted to 50 ml with phosphate buffer.

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## Procedure

Pipetted out 2 ml of phosphate buffer, 1 ml of para coumaric acid, 1 ml of manganese chloride and 2 ml of enzyme extract. Started the reaction by adding 4 ml of IAA solution. Incubated the reaction mixture in the dark with shaking at 30°C. Withdrew 2 ml of the mixture after 0 and 50 minutes of incubation and added 5.2 ml perchloric acid and 0.5 ml ferric nitrite solution. Then diluted to 10 ml with water. After incubating the reaction mixture in the dark for 60 minutes measured the absorbance at 535 nm.

## Appendix 11

### Estimation of polyphenol oxidase activity (Bateman and Daly, 1967) Spectrophotometric Method

The intensely yellow 2-nitro-5-thiobenzoic acid (TNB) with an absorption maximum at 412 nm reacts with the quinones generated through enzymatic oxidation of 4-methylcatechol (catechol oxidase) and 1,4 dihydroxybenzene (laccase) to yield colourless adducts. The decrease in the absorbance of yellow colour due to enzyme activity is measured using the reagents namely 0.2 M citrate phosphate buffer, 2-nitro-5-thiobenzoic acid anion, quinine solutions, substrate solution – 4 methylcatechol (2 mM) for catechol assay Quinol (1,4 dihydroxybenzene, 2 mM) for laccase assay, Enzyme extract: Prepared first acetone powder of fresh plant tissue. To get a crude enzyme preparation, mixed 100mg acetone powder with 2.5 ml of 0.2 M citrate phosphate buffer, 1 ml of Triton X 100, 6.5 ml of water and 500 mg polyamide. Shook for 1hr and filtered. Used the filtrate as enzyme source.

## Procedure

Pipetted out into clean 1cm cuvette of 1.4 ml citrate 0.1 M phosphate buffer, 0.5 ml of TNB and 1 ml of the substrate solution. The reaction was initiated by the addition of 0.1 ml of enzyme preparation and immediately noted down the absorbance at 412 nm in a spectrophotometer already set. Followed the decrease in absorbance at 30 seconds intervals and recorded.

## Appendix 12

### Estimation of nitrate reductase activity (Nicholas *et al.*, 1976) Colorimetric Method

Nitrate reductase (NR) is capable of utilizing the reduced form of pyridine nucleotides, flavins or benzyl viologen as electron donors for reduction of nitrite. NADH-dependent nitrate reductase is most prevalent in plants. Hence, NR activity in plants can be measured by following the oxidation of NAD (P) H at 340 nm. However,

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NR activity is commonly measured by colorimetric determination of nitrite produced and the reagents used were 0.1M potassium phosphate buffer (pH 7.5), 0.1 M potassium nitrate, 2mM NADH, 1% sulphanilamide, 0.02% N-(1-naphthyl)ethylenediamine dihydrochloride, 0.01 M potassium nitrite standard solution. Enzyme extract: Homogenized a weighed quantity of the plant material in a known volume of medium containing 1 mM EDTA, 1-25 mM cysteine and 25 mM potassium phosphate adjusted to a final pH 8.8 with potassium hydroxide. Filtered through four layers of cheese cloth and centrifuged for 15 minutes at 30,000g. Decanted the supernatant through glass wool and used for assays. Extracted under ice cold conditions.

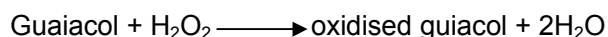
### **Procedure**

Pipetted out 0.5 ml phosphate buffer (pH 7.5) in a test tube. Added 0.2 ml potassium nitrate solution, 0.4 ml NADH solution and 0.7 ml water. Initiated the reaction by the addition of 0.2 ml enzyme extract. Set up a control in the same way but with water instead of enzyme extract. Incubated at 30°C for 15 minutes. Terminated the reaction by the rapid addition of 1 ml sulphanilamide followed by 1 ml naphthyl ethylenediamine reagent. Waited for 90 minutes and measured the absorbance at 540 nm. A standard graph was prepared with sodium nitrite. Pipetted out different known aliquots of potassium nitrite standard solution into a series of test tubes and made up the volume in each tube to 2 ml by adding water and proceeded as for sample. Activity is expressed as micromole nitrite produced per minute per mg protein.

## **Appendix 13**

### **Estimation of peroxidase activity (Perur ,1962 and Angelini *et al.*, 1990) Spectrophotometric Method**

Guaiacol is used as substrate for the assay of peroxidase.



The resulting oxidized guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the POD activity and can be assayed spectrophotometrically at 436 nm using the reagents namely 0.1 M phosphate buffer, 20 mM Guaiacol solution and 12.3 mM hydrogen peroxide solution.

Enzyme extract: Extract of 1 g of fresh plant tissue in 3 ml of 0.1 M phosphate buffer pH 7 by grinding in a pre cooled mortar and pestle. Centrifuged the homogenate at 18,000 g at 5°C for 15 minutes. Used the supernatant as enzyme source within 2-4 h. Stored on ice till the assay was carried out.

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**Procedure**

Pipetted out 3 ml buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide solution in a cuvette. Mixed well. Placed the cuvette in the spectrophotometer. Waited until the absorbance has increased by 0.05. Started a stop watch and noted time required in minutes ( $\Delta t$ ) to increase the absorbance by 0.1. Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified is 6.39 per micromole, the enzyme activity per litre of extract is calculated as

$$\text{Enzyme activity units / litre} = \frac{3.18 \times 0.1 \times 1000}{6.39 \times 1 \times \Delta t \times 0.1} = 500 / \Delta t$$

**Appendix 14****Estimation of acid phosphatase activity****(Tominaga and Takeshi, 1974)****Colorimetric Method**

The enzyme phosphatase hydrolyses p-nitrophenol phosphate. The released p-nitrophenol is yellow in colour in alkaline medium and is measured at 405 nm. The optimum pH for acid phosphatase is 5.3. The reagents used were 0.085 N sodium hydroxide, substrate solution, standard solution of p-nitrophenol.

Enzyme extract: Homogenised 1 g of fresh tissue in 10 ml of ice cold 50 mM citrate buffer in a prechilled pestle and mortar. Filtered through four layers of cheese cloth. Centrifuged the filtrate at 10,000g for 10 minutes. Used the supernatant as enzyme source.

**Procedure**

Incubated 3ml of substrate solution at 37° C for 5 minutes. Added 0.5 ml enzyme extract and mixed well. Removed immediately 0.05 ml and mixed it with 9.5 ml of 0.08 N sodium hydroxide. This corresponds to zero time assay (blank). Incubated the remaining solution for 15 minutes at 37° C. Drew 0.5 ml sample and mixed it with 9.5 ml sodium hydroxide solution. Measured the absorbance of blank and incubated tubes at 405 nm. Took 0.2-1.0 ml of standard, diluted to 10 ml with sodium hydroxide solution. Read the colour and drew the standard curve.

**Appendix 15****Estimation of catalase activity****(Gopalachari, 1963)****Colorimetric Method**

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250 nm. On decomposition of hydrogen peroxide by catalase, the

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absorption decreases with time. The enzyme activity could be arrived at from this decrease. The reagents used were 0.067 M phosphate buffer, hydrogen peroxide-phosphate buffer, Enzyme extract: Homogenized plant tissue in a blender with M/150 phosphate buffer at 1-4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allow standing in the cold with occasional shaking and then repeating the extraction once or twice.

### **Procedure**

Read at 240 nm against a control cuvette containing enzyme solution as in the experimental cuvette, but containing hydrogen peroxide free phosphate buffer (M/15). Pipetted into the experimental cuvette, 3 ml hydrogen peroxide buffer. Mixed in 0.01-0.04 ml sample with a glass or plastic rod flattened at one end. Noted the time  $\Delta t$  required for a decrease in absorbance from 0.45-0.4. This value was used for calculations. If it was greater than 60 sec repeated the measurements with a more concentrated solution of the sample.

## **Appendix 16**

### **Estimation of nitrogen**

**(Humphries, 1956)**

#### **Micro-Kjeldahl Method**

The nitrogen in protein or any other organic material is converted to ammonium sulphate by sulphuric acid during digestion. This salt, on steam distillation, liberates ammonia which is collected in boric acid solution and titrated against standard acid. Since 1 ml of 0.1 N acid is equivalent to 1.401 mg nitrogen, calculation is made to arrive at the nitrogen content of the sample. The reagents used were sulphuric acid, mercuric oxide, potassium sulphate, sodium hydroxide-sodium thiosulphate solution, indicator solution, 4% boric acid solution, standard hydrochloric acid and boiling chips.

### **Procedure**

Weighed 100 mg of the sample and transferred to a 30 ml digestion flask. Added  $1.9 \pm 0.1$ g potassium sulphate and  $80 \pm 10$  mg mercuric oxide and 2 ml concentrated sulphuric acid to the digestion flask. If sample size was larger than 20 mg dry weight, 0.1 ml sulphuric acid should be added for each 10 mg dry material. Added boiling chips and digested the sample till the solution becomes colourless. After cooling the digest, dilute it with a small quantity of distilled ammonia free water and transferred to the distillation apparatus. The kjeldahl flask should be rinsed with successive small quantities of water. Placed a 100 ml conical flask containing 5 ml of boric acid solution with a few drops of mixed indicator with the tip of the condenser dipping below the surface of the solution. Added 10ml of sodium hydroxide- sodium thiosulphate solution to the test solution in the apparatus. Distilled and collected the ammonia on boric acid. Rinsed the tip of the condenser and titrated the solution against the standard acid until appearance of violet colour, the end point. Run a blank.

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**Appendix 17**  
**Estimation of phosphorus**  
**(Jackson *et al.*, 1973)**  
**Spectrophotometric Method**

The phytate is extracted with trichloroacetic acid and precipitated as ferric salt. The iron content of the precipitate is determined spectrophotometrically and the phytate phosphorus content calculated from this value assuming a constant 4 Fe:6 P molecular ratio in the precipitate. Weighed a finely ground sample and extracted in 3% TCA, centrifuged the suspension and transferred the supernatant. Added ferric chloride and heated the contents, centrifuged. Washed the precipitate in 3% TCA and heated. Dispersed in water and added 1.5N sodium hydroxide. Heated and filtered. Dissolved the precipitate with 3.2N nitric acid. Washed with water and collected the washings. Cooled and added 1.5 M KSCN, read the colour within a minute at 480 nm. Run a blank simultaneously. (Ferric nitrate was used as standard).

**Appendix 18**  
**Estimation of sodium and potassium**  
**(Jackson *et al.*, 1973)**  
**Flame Photometric Method**

In flame photometry, the solution under test is passed under carefully controlled conditions as a very fine spray in the air supply to a burner. In the flame, the solution evaporates and the salt dissociates to give neutral atoms. A very small proportion of these moves into a higher energy state. When these excited atoms fall back to the ground state, the light emitted of characteristic wavelength is measured. Sodium and potassium are estimated at 590 and 770 nm respectively using reagents namely sodium stock solution: 2.524 g of sodium chloride was dissolved in deionised distilled water and made upto one litre (1ml = 1.0 mg sodium), potassium stock solution: 1.907 g of potassium chloride was dissolved in deionised distilled water and made upto one litre (1ml = 1.0 mg potassium).

**Procedure**

Standardized the flame photometer before feeding the sample. Set the reading to zero using deionised water. Using the stock solutions of sodium and potassium, adjusted the reading to 100 at their specific wavelengths. Then fed the sample in the flame photometer and noted the readings which gave the amounts of sodium and potassium directly.

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**Appendix 19**  
**Estimation of calcium and magnesium**  
**(Jackson *et al.*, 1973)**  
**Versanate Method**

Ethylene diamine tetra acetic acid (EDTA) forms stable complex with various polyvalent cations at different pH levels. Calcium and magnesium get complexed by EDTA in the order calcium first and magnesium afterwards at different pH levels of 12 and 16 respectively. Hence calcium was first estimated by titration with EDTA using murexide as indicator in the presence of sodium hydroxide at pH 12. Then magnesium was estimated together with calcium using Erichrome black T indicator at pH 10 in the presence of ammonium chloride and ammonium hydroxide buffer solution using reagents namely 10% sodium hydroxide solution, murexide indicator (ammonium purpurate), ammonium chloride – ammonium hydroxide buffer solution, Erichrome black T indicator : 0.5 g of the dye was mixed with 100 g of sodium chloride to obtain a dry powder mixture, standard EDTA titrant 0.02 N : 3.723 g of disodium ethylene diamine tetra acetate dehydrate was dissolved in one litre of water. It was standardized against standard calcium solution (1ml of 0.02 N EDTA  $\equiv$  1.0 mg calcium carbonate), calcium standard solution: 1.0 g calcium carbonate was dissolved in one litre of water using 20.5 ml of 1 N hydrochloric acid by warming.

**Procedure**

**Calcium alone:** Pipetted out 25 ml of water into a porcelain dish. Added 10 ml of sodium hydroxide solution. Put a red litmus paper and tested whether it turned into blue or not. If there was no change, added some more alkali till the red litmus paper turned blue in colour. Then added a pinch of murexide indicator. Titrated this against 0.02 N EDTA till the colour changed from pink red to violet.

**Calcium + Magnesium:** Pipetted out 25 ml water into a porcelain dish. Put a red litmus paper and added ammonium chloride-ammonium hydroxide buffer solution till the litmus paper turned into blue. Then added two drops of erichrome black T indicator. Titrated this against 0.02 N EDTA solution till the colour changed from purplish red to sky blue colour.

**Appendix 20**  
**Gene expression analysis of heat shock protein-70 (Hsp-70)**  
**in the plant tissue**

Gene Expression Analysis of Heat Shock Protein-70 (HSP-70) in plant leaf tissue using Tubulin gene as endogenous control.

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## Primers used

### Set Primer Sequence (5'-3')

1. **HS1f** GGA TTT CGA CAA CAG GAT GG  
**HS1r** CCT TCT CGT TAC CCT CAC CA

2. **TubA1f** GTG CAT TTC AAT CCA CAT CG  
**TubA1r** CAG TGG GCT CAA GAT CAA CA

## Instruments

RNA Isolation – Trizol Reagent (Invitrogen), RNA Electrophoresis, RevertAid H Minus First Strand cDNA Synthesis Kit (MBI-Fermentas), 2 X PCR Master Mix – (MBI-Fermentas), Quantifast SYBR GREEN PCR kit- Qiagen, NanoDrop 8000 Spectrophotometer, Agarose Gel Electrophoresis, Mastercycler ep Gradient Real Plex Thermalcycler ep Gradient, ChemiDoc XRS Gel Quantification.

## Procedure

### RNA Isolation

#### Total RNA isolated from leaf tissue

100 mg of tissue was crushed in liquid nitrogen using mortar and pestle and tissue was converted into powder form. 1 ml of Trizol reagent was added to tissue powder, mixed and incubated in ice for 15 min. The tube was centrifuged at 5000 rpm for 5 minutes, supernatant was transferred in new 1.5 ml tube and pellet was discarded. 200 µl of chloroform was added to supernatant, mixed by vortexing and incubated on ice for 10 min. For phase separation the tube was centrifuged at 13000 rpm for 15 min. Upper clear aqueous phase was transferred in new 1.5 ml tube and 500 µl of isopropanol was added. The tube was incubated in ice for 15 min and centrifuged at 13000 for 15 min. Isopropanol was discarded and the formed RNA pellet was washed with 70 per cent ethanol prepared in DEPC treated water. Tube was centrifuged at 13000 rpm for 30 min. Ethanol was decanted and tube was kept at room temperature for 15-20 min for pellet drying. Dried pellet was dissolved in 30 µl of DEPC treated water and tube was incubated at 65°C for 15 minutes. The RNA stock sample was quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 40 µg RNA per ml.

### Denaturing Agarose Gel Electrophoresis

1 gm of agarose was heated in DEPC treated water, cooled to 60°C, and 5 ml of 10 X MOPS buffer was added followed by 37% formaldehyde. This was then mixed properly and casted in the AGE unit. RNA sample (2 µl) was mixed with 2 µl of 2X RNA loading dye, heated at 70 °C for 5 minutes, chilled on ice for another 5 minutes and mixed with 0.5 µl of 1% EtBr and loaded on the gel. The electrophoresis was carried

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out using 1X MOPS buffer. The image was captured using the ChemiDoc XRS instrument.

### **DNase treatment of RNA**

Following is reaction for DNase treatment: Total RNA 10 µl, 10 reaction buffer 2 µl, DNase 1 3 µl, DEPC treated water 5 µl. Incubate mixture at 37°C for 30 min and added 2 µl of 25 mM EDTA and incubated at 65°C for 10 minutes.

### **First cDNA synthesis using RevertAid H Minus First Strand cDNA Synthesis Kit**

cDNA is prepared for two samples using H minus First Strand cDNA synthesis using the Random Hexamers primers. The following components were added in 0.2 PCR microcentrifuge tube in the order kept on ice: Total RNA Template- 4 µl, Primer (Random Hexamers)-1 µl, DEPC -treated water-7 µl, 5x Reaction Buffer- 4 µl, RiboLock RNase Inhibitor (20u/ul)-1 µl, 10 mM dNTP mix- 2 µl, M-MuLV Rev. Transcriptase (20u/ul)-1 µl.

Mixed gently, centrifuged and incubated for 5 minutes at 25°C followed by 60 minutes at 42 °C. The reaction was terminated by heating at 70 °C for 5 minutes and kept hold at 4°C.

### **PCR Amplification of HSP-70 and Tubulin gene from cDNA**

HSP-70 and tubulin gene were amplified from prepared cDNA using gene specific primers for optimization of cycling conditions and primer to template ratio.

### **PCR reaction set up for amplification of cDNA**

Initial- Denaturation- 95°C - 8 min, Denaturation- 95°C- 30 sec, Annealing- 46°C- 30 sec- 43 x, Extension- 72°C- 35 sec, Final Extension- 72°C- 10 mins.

### **2% Agarose Gel Electrophoresis for quantification of PCR products**

1 gm of Ultrapure Agarose was heated in 50 ml 1 x TAE, cool to 60 °C and 1% Ethidium bromide was added at 10µl /100ml concentration. 5 µl of PCR product electrophoresed along with 1kb DNA molecular weight marker (Zipruler1 Fermentas).Electrophoresis was carried out at 80V for 30 min at room temperature.

### **Real Time PCR**

In the present assay, the groundnut leaves from salt treated and control plants were relatively quantified using real time PCR with HSP-70: Heat Shock Protein-70 (test gene) and TUB: Tubulin gene (Reference gene). Real Time PCR reaction for both genes of both samples was performed in triplicate using Quantifast SYBR GREEN Real Time PCR Master mix.

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**Real Time PCR set up for relative quantification assay steps**

Initial- Denaturation- 95°C- 8 min- 1x, Denaturation- 95°C- 30 sec, Annealing- 60°C- 30 sec- 43 x, Extension- 72°C- 35 sec, Final Extension- 72°C- 10 min- 1x

Melt Curve 95°C- 15 sec, 60°C- 15 sec- 1x, 20 min, 95°C- 15 sec.

Following are formulae utilized in calculation of fold of expression

**$\Delta CT = CT_{\text{target}} - CT_{\text{House keeping}}$**

□  **$\Delta \Delta CT = \Delta CT_{\text{Test samples}} - \Delta CT_{\text{Calibrator samples}}$**

□ **Fold difference =  $2^{-\Delta \Delta CT}$**