CHAPTER III

MATERIALS AND METHODS
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The study was conducted at the Biotechnology Laboratory of the Division of Horticulture, ICAR Research Complex for NEH Region, Barapani, Meghalaya, India.

3.1. Cultivars for study

Four economically important Citrus species viz., Khasi Mandarin (Citrus reticulata Blanco) [KM], Kinnow (Citrus nobilis × Citrus deliciosa Tenore) [KIN], Citrus volkameriana Tan and Pasq [CV] and Citrus reshini Tanaka [CLM] were selected for study.

3.1.1. Explant for micropropagation

Explant for micropropagation were taken from nucellar seedlings (Through nucellar embryogenesis) and Shoot tip.

3.2. Standardisation of media

Murshige and Skoog (MS) media (1962) and Gamborg (1968), Nitsch (1951) and White’s (1954) with slight modification where ever required were used in the investigation as basal media. The required plant growth substances such as benzyl amino purine (BAP), paclobutrazol, antitranspirants were used in various concentrations. Most of the chemical constituents used for the preparation of media were analytical grade.

a. Composition of media

Full details of media constituents are given in appendix - I. The required quantities of macro and micronutrients were weighed in analytical electronic balance and dissolved in double distilled water. The stocks were prepared as 10 X concentration and stored in reagent bottles. Stock solutions were also prepared for myoinositol, glycine, vitamins and for each hormone required for establishment of cultures, initiation of growth and maintenance, proliferation and rooting. Stock
solution were stored in refrigeration until use and subsequently supplemented to basal media at different concentrations. Paclobutrazol was added to the medium for rooting. Antitranspirants were sprayed over plants after transferring them into bottles.

b. Media preparation and sterilization

To prepare one litre of medium, required amount of each of the macro and micro elements, vitamins, hormone, a carbon source and any other supplements were added as appropriate and final volume was made upto 1 litre by adding double distilled water. The pH was adjusted at 5.8 using 0.1 N KOH or HCl. Agar (0.8% W/V) was dissolved in the medium for semi solid media. The media were dispensed into culture tubes and sterilized by autoclaving at 121°c with 1.8kg/cm² pressure for 20 minutes after capping. Alternatively, media containing heat labile components were prepared by filter sterilization of the components using millipore/ tarson sterile filtration system and added to sterile media in aseptic conditions. The media were dispensed into test tubes and used after 48 hours.

c. Aseptic inoculation

All the steps i.e., surface sterilization of explants, preparation and inoculation and further subculturing were carried out in a sterile laminar air flow cabinet. Before using the laminar air flow, it was sprayed with alcohol using a hand sprayer and wiped out with cotton dipped in alcohol. All the surgical instruments and tiles were autoclaved and before using they were dipped in alcohol, flammed and cooled.

d. Culture condition

The cultures were generally maintained in dust free air conditioned room at 25 ± 2°c and relative humidity of around 60% under a photoperiodic regime of 16 hours light and 8 hours dark cycles provided by fluorescent tubes (40W, 220 V) at an intensity of 3000 lux at the culture level.
3.2.1. Preparation of explant

a. Culture initiation

For obtaining nucellar seedlings, methods described by Parthasarthy and Nagaraju (1994) was followed. Ovules were excised from 8 to 10 weeks old fruits. The fruits were immersed in ethanol for 2 mins and the ovules were excised aseptically and cultured in MS medium supplemented with malt extract (500 mg/l). Shoot tip explant from seeding plants were cultured in MS medium after sterilizing with 0.01 % mercuric chloride for 5 mins and subsequently 2 to 4 times washing with sterile distilled water. Cultures were incubated in dark room for 48 hours and then brought to light.

b. Shoot proliferation

Shoot tip obtained in vitro were used as explant for further shoot proliferation studies. Individual shoot were trimmed to 0.5 to 1 cm long on sterile tile in a laminar air flow cabinet using sterile scalpel and forcep and cultured in MS medium supplemented with BAP concentration of 0.25 mg/l to 0.5 mg/l. The cultures were maintained in culture room.

3.3. In vitro hardening

In vitro proliferated shoots trimmed to 1 cm long were inoculated in basal media supplemented with sucrose (7.5, 15, 30 and 60 g/l) and Paclobutrazol (0.25, 0.5, 0.75, 1.0, 2.0, 3.0 and 4.0 mg/l) for rooting and kept in culture room at 25 ± 2° under 3000 lux in 16 hours photoperiod. Observations were recorded after 4 weeks of culture.

3.4. Reduced humidity and antitranspirants

In vitro rooted shoots were transferred to soilrite in sterilize bottle and relative humidity inside the bottle was reduced using silica gel (8g). Silica gel was tied with perforated cloth and suspended inside the bottle with cotton head. Antitranspirants used were alar (1mg/l and 2 mg/l) and 8 Hydroxi Quinolin (1 mg/l and 2 mg/l). Antitranspirants were sprayed over plants at two days interval.
Observations were recorded after 4 weeks of culture and then transferred to mist house. The relative water content (RWC) was estimated following the methods of Barrs and Weatherly (1962).

3.5. **Simultaneous rooting and acclimatization**

Microshoots of about 2-3 cm long were harvested from proliferating cultures of four *Citrus* species and directly cultured in tissue culture bottle (450 ml capacity) containing different carriers *viz.*, soilrite (SR), farm yard manure (FYM), garden soil (GS), sand (SD), SR + FYM, SR + GS, SR + SD, FYM + GS, FYM + SD, GS + SD. These bottles were filled up to one third with these carriers and one fourth strength of MS (1962) salt solution added depending on its capacity of absorption before autoclaving at 15 lb for 20 minutes. 3 to 4 microshoots were taken in each bottle and 30 bottles were used in each treatment. These bottles were placed in culture room for one week. Then they were transferred to mist house under shade (75% shade net). Caps were loosened gradually and subjected to exposure to greenhouse environment once in a week. Observations were recorded after 4 weeks and then transferred to polythene bag containing 1:1 soil and FYM.

3.6. **Transfer of plants to greenhouse**

Plantlets rooted in varying concentration of sucrose and paclobutrazol were transferred after four weeks of culture to bottle containing sterile soilrite and placed in the culture room for one week and then transferred to greenhouse. Plants treated with silica gel and antitranspirants were kept in culture room for 4 weeks and then transferred to mist house. Similarly plants grown in different carriers were also placed in culture room for 4 weeks and then transferred to greenhouse. Before transferring to greenhouse, caps were loosened gradually and then plants were planted in polythene bag containing 1:1 soil and FYM. Regular monitoring was done. *Ex vitro* survival percent was recorded after 4 weeks in the mist house.
3.7. Biochemical analysis

a. Preparation of alcohol extract

Shoots, leaves and root sample (1 g) collected at various stages were cut into small pieces and crushed with 5 ml of hot ethanol with pestle and mortar and filtered through at two layers of chees cloth. The extract collected was used for estimating alcohol soluble solid such as total sugar, reducing sugar, total phenol and OD phenol. The residue left in the cheese cloth was oven dried and used for determining total protein and starch.

3.7.1. Estimation of reducing sugars (Nelson, 1944)

Reagents.

Copper reagent A: Prepared using 25 g of anydrous sodium carbonate, 25 g sodium potassium tartrate (Rachelle salt), 20 g sodium bicarbonate and 200 g anhydrous sodium sulphate was dissolved in 800 ml double glass distilled water and volume was made upto 1000 ml.

Copper reagent B: 15 g copper sulphate was dissolved in 100 ml of double glass distilled water containing 1-2 drops of concentrated sulphuric acid.

Arsenomolybdate colour reagent: 25 g of ammonium molydate was dissolved in 450 ml of double glass distilled water and 21 ml of conc. sulphuric acid was added and mixed. 3 g of sodium arsenate dissolved in 25 ml distilled water was added and incubated at 37°C for 48 hours in amber coloured bottle.

Procedure: 0.05 ml of aliquot was taken and made up to 1 ml in a graduated narrow tube using ethanol. 1 ml of mixture prepared fresh using 25 parts reagent A to a part reagent B was added to the extract and placed the tube on boiling water bath for 20 minutes, then cooled under a running tap and 1 ml of arsenomolybdate reagent was added and after 15 minutes the mixture was diluted to 25 ml graduation and absorbance was measured at 500 nm using spectrophotometer after adjusting the absorbance to zero using blank. Amount of reducing sugars present in the unknown sample was calculated using a glucose standard curve obtained using different concentrations.
3.7.2. *Estimation of total phenols* (Bray and Thorpe, 1954)

**Reagents**

Folin-ciocalteu reagent

20% sodium carbonate

**Procedure:** 1 ml of aliquot was pipetted to narrow graduated test tube to which 1 ml of Folin-ciocalteu reagent followed by 2 ml of 20% sodium carbonate was added and kept on a boiling water bath after mixing gently. After 1 minute the tube was taken out and cooled under a running tap water and diluted the blue coloured solution to 25 ml with distilled water and recorded the absorbance at 650 nm using spectrophotometer, after adjusting the absorbance to zero using a blank and amount of total phenols present in a sample was calculated using a standard curve obtained using a different concentrations of catechol.

3.7.3. *Estimation of ortho-dihydric (OD) phenols* (Mahadevan, 1966)

**Reagents**

HCl. 0.5 N

NaOH 1 N

**Procedure:** To 1 ml of aliquot, 1 ml of 0.5 N HCL, 1 ml of Arnow's reagent, 10 ml of distilled water and 2 ml of 1 N NaOH was added. Immediately after adding alkali pink colour solution was formed and its absorbance was recorded at 515 nm using a spectrophotometer after adjusting the absorbance zero using a blank. Amount of OD phenols present in sample was calculated using a standard curve preparation with catechol.

3.7.4. *Determination of protein* (Lowry et al., 1951)

**A. Separation precipitation and clarification:**

**Reagents**

Absolute and 80% ethanol

Ethanol-Enter mixture (3 : 1 v/v)

TCA 5%

**Method:** After extraction of alcohol slouble solides using boiling ethanol, the residue left in the muslin cloth was oven dried and 25 mg
of powdered sample was taken in a cold centrifuge tubes and to this tube 5% cold TCA was added and kept in an ice bath for 15 minutes then centrifuged at 2000 g for 20 minutes and supernatant was discarded and this process was repeated twice. The pellet was then re-extracted once with absolute ethanol and twice with 3:1 hot ethanol : ether mixture evry time discarding the supernatant and the pellet was used for protein determination.

B. Protein determination

Reagents

Alkaline sodium carbonate solution : 2% sodium carbonate in 0.1 N NaOH (1)

0.5% copper sulphate : 1% sodium potassium tartrate solution (2)

Alkaline copper reagent : This reagent was freshly prepared using 50 ml of reagent 1 with 1 ml of reagent 2.

Folin-ciocalteu reagent : Commercially available reagent was diluted with equal volume of distilled water.

1 N NaOH.

Procedure : The residue left in the centrifuge tube after clarification was placed in 1 ml of 1 N NaOH at 100°C for 4-5 minutes and 5 ml of alkaline copper reagent was added and allowed to stand at room temperature for at least 10 minutes. 0.5 ml of Folin-ciocalteu reagent was added rapidly and mixed immediately. After 20 minutes, absorbance was measured at 750 nm using spectrophotometer after adjusting the absorbance to zero using blank. Using standard of Bovine serum albumin amount of preteins present in the aliquot was calculated.

3.7.5. Estimation of Starch (Mahadevan and Sridhar, 1986b)

Reagents :

Ethanol 80%

Perchloric acid 52%

Anthrone reagent : 2 g of anthrone was dissoved in 1 litre of concentrated sulphuric acid on the day of use.
Procedure: 25 mg of dried and ground alcohol insoluble solid sample was taken in a centrifuge tube and 80% ethanol was added and kept in a water bath at 80°C for 10 minutes and centrifuged 3 times at 2000 g for 15 minutes to remove any alcohol soluble sugars. The residue left was used for starch estimation as described below.

5 ml of distilled water and 6.5 ml of 52 per cent perchloric acid was added to the residue and stirred constantly with the help of a glass rod for 5 minutes then occasionally for 15 minutes. 5 ml of distilled water was added and centrifuged at 2000 g for 15 minutes and supernatant was transferred to 25 ml volumetric flask. 5 ml of distilled water and 6.5 ml of perchloric acid was added again and contents were stirred occasionally for next 30 minutes and transferred to volumetric flask and volume was made upto 25 ml using distilled water and filtered through Whatman No. 42 filter paper. First few ml of filtrate was discarded and 0.1 ml of filtrate was taken in a test tube and made upto 1 ml using distilled water and 4 ml of anthrone reagent was added through the sides of the tube and closed with glass marble and kept in boiling water bath for 10 minutes and cooled to room temperature using a cold water bath and the blue green coloured solution obtained was measured at 625 nm in spectrophotometer after adjusting the absorbance to zero using a blank. Amount of starch present in an aliquot was measured using standard curve obtained using glucose and multiplied by factor 0.9.

3.8. PHYSIOLOGICAL STUDY

3.8.1 Stomatal index

Stomatal index was determined following the method of Dhawan and Bhojwani (1987).

Procedure: Dorsal surface of leaves were painted with slurry obtained by mixing xylene with thermocol. After the mixture is dried, it was peel as ribbon and mounted in a drop of water on a microscope slide. The number of stomata and epidermal cells per unit area were determined, from the surface of leaflets using the 40x objectives and
10x eyepiece of a Bausch and Lomb microscope, Stomatal index (SI) was calculated as follows:

\[
SI = \frac{NS}{NS + NE} \times 100
\]

Where, NS is the number of stomata
NE is the number of epidermal cells.

3.8.2 Chlorophyll content of leaf (Singh, 1982)
Requirements: Leaves, 80% acetone (v/v), spectrophotometer or colorimeter, Buchner funnel, mortar and pestle.
Procedure: Grind 1 gm tissue with 40 ml of 80% acetone to a fine pulp. Transfer the resulting green liquid to a Buchner funnel lined with Whatman No. 1 filter paper. Repeat the grinding of the pulp several times with a fresh 30 ml aliquot of 80% acetone. Filter the extract each time as before into the same flask containing first extract. Make up the volume of the filtrate to 100 ml by adding sufficient 80% acetone. Chlorophyll determination: Record the optical density (OD) of the chlorophyll extract is in a 10 mm cell with a spectrophotometer set at 652 nm against 80% acetone solvent blank. Calculate the amount of chlorophyll present (in mg/gm of tissue) in the extract according to the following equation:

\[
\text{Total Chl.} = \frac{OD \times 1000}{34.5} \times \frac{V}{1000 \times W}
\]

Where, OD = optical density.
\(V\) = the final volume of the 80% acetone chlorophyll extract
\(W\) = fresh weight in grams of the tissue extracted.

3.8.3. Epicuticular wax

Epicuticular wax was determined by the method of Ebercon et al. (1977) based on the colour change produced due to the reaction of wax with acidic potassium bichromate.

The reagent was prepared by mixing 40 ml of deionized water with 20 g of powdered potassium bichromate. The resulting slurry was mixed vigorously with 1 litre concentrated sulfuric acid and heated
The individual sample consisted of 10 leaf disc having area of 30.8 cm$^2$. Each sample was immersed in 15 ml redistilled chloroform for 15 seconds. The extract was filtered and evaporated on a boiling water bath, with the smell of chloroform could not be detected. After adding 5 ml of reagent, samples were placed in boiling water for 30 min. After cooling 12 ml of deionized water was added. Several minutes were allowed for colour development and cooling and then the optical density of the sample was read at 590 nm.

3.8.4. SCANNING ELECTRON MICROSCOPY OF LEAF

Scanning of leaf carried out in the Regional Sophisticated Instrumentation Centre, NEHU, Shillong. The leaf discs of about 0.2 cm diameter were immersed in 4% formalin and kept overnight at room temperature. After 12 hours leaf discs were washed with acetone of different concentrations (30%, 50%, 70%, 80%, 95% and dry acetone). Two washings of same concentrations were done with an interval of 15 minutes. Then samples were dried, mounted for gold coating and prepared for scanning electron microscopy under a scanning electron microscope (JEOL, 35 CF, Japan) as the method described by Dey et al. (1989). Photographs were taken at different magnitude.

3.9. STATISTICAL ANALYSIS

3.9.1. Analysis of variance

To test the differences among treatments in the experiments conducted, the data obtained for each character were analysed by following randomized complete block design analysis or factorial analysis as the case may be. The analysis was based on following linear model given by Fisher (1958).

$$Y_{ij} = \mu + b_i + t_j + e_{ij}$$

Where,

- $Y_{ij}$ = Performance of $j^{th}$ genotype in the $i^{th}$ block
- $\mu$ = General mean
- $b_i$ = true effect of $i^{th}$ block
The principal component were calculated as per the method described by Adams and Wiersma (1978) as given below.

Consider a set of n treatments, each of which has been scored for several, m metrical traits. With the m variables as coordinate axes, the treatments may be positioned in the multi dimensional space, their pattern of dispersion resembling the multi dimensional analogue of an ordinary 2 way scattergram. When the raw data are untransformed and with correlation among the variables, the multidimensional scattergram will resemble an ellipsoidal configuration comprise the principal components in a principal component analysis.

PCA consists of a standardization and orthogonal angular rotation of the original axes (variables) to a new set of axes, the principal components. Each principal component is in reality a linear combination of the treatment scores on the treatment scores on the original variables. A set of m homogeneous equations in m unknown is generated, expressible ultimately in matrix form as

$$(R_c - \lambda I) = 0$$

Where, $\lambda$ = the diagonal matrix of the latent roots (Latent roots are also known as eigenroots or eigen values. There are m roots, the first root is equivalent to the variance accounted for by the first principal component the second root is the variance attributable to the second principal component and so on to the mth root and component).

$\mathbf{b}$= matrix of latent vectors (Latent vectors are also known as eigenvectors, and comprise the orthogonal transformation matrix by which the original standardized variates must be multiplied to produce
the transformed variates.

\[ I = \text{the identity matrix and} \]
\[ R_c = \text{the matrix of correlation coefficients between pairs of variables.} \]