CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of the four citrus species citrumelo (*Poncirus trifoliata*×*C. paradisi*), limau langkat (*C. suhuiensis*), rangpur lime (*C. limonia*), and cleopatra (*C. reticulata*) were collected from Dr. Shahrudin Saamin of Malaysian Agriculture Research And Development Institute (MARDI) Cameron Highlands and grown aseptically.

Seeds were cultured aseptically on MS basal medium and plants were maintained by subculturing 2 shoot tips or nodes (2 cm) to 35 - 40 ml of the same medium every 8-10 weeks.

2.1.1. Seed sterilisation

The seeds were washed thoroughly under running water for 13 to 14 h. to remove the residue of fruit vesicles and gelatin substances that surrounded the seeds. After a final rinse in distilled water, they were transferred to the laminar flow cabinet. Seed coats were removed and the seeds were sterilised by immersing in ethanol 70 % (v/v) for 30 sec., followed by continuous shaking for 20 minutes in 1% (w/v) sodium hypochlorite with the addition of 0.1% (v/v) Tween-20 and subsequently rinsed 3-4 times in autoclaved distilled water. ~~The seed coats were removed in aseptic conditions in order to accelerate germination~~. Three to four seeds were cultured on MS basal medium (Appendix A) dispensed in 500 ml jars. Cultures were maintained as in 2.1.2 .

2.1 .2 . Culture conditions

All cultures were maintained under continuos cool-white fluorescent light (50 μmol m^{-2} s^{-1}) at 26 ± 1 °C.

2 . 2 . Culture media

For the following tissue culture experiments, Murashige and Tucker (1969), (MT) was used as basal medium (Appendix A). Two growth regulators, namely 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) were incorporated individually or in different combinations (Appendix A). The media were dispensed in sterile plastic vials containing 20 to 25 ml of culture medium. Three to four explants were cultured per bottle and 10 to 15 replicates were used from each type of explants. All cultures were maintained under the same cultural conditions as in 2.1.2 .

2. 3. Preparation of explants

Explants from the four species of citrus were taken from 10 to 14 week-old *in-vitro* grown seedlings.

2. 3. 1. Nodal explants

Nodal pieces each bearing one axillary bud were implanted on MT basal medium (1969) (Appendix A) supplemented with two phytohormones (NAA and BAP) for proliferation of shoots and / or root assessments .

2. 3. 2. Stem (internodal) explants

Internodal stem tissue were cut into segments (approximately 0.5 cm in length) and cultured on MT basal media with different levels of phytohormones (Appendix A). Three to four explants were cultured in each sterile plastic vial containing about 20 ml of culture media.

2. 3. 3. Leaf explants:

Fully grown leaves from *in-vitro* grown seedlings were cut into pieces (approximately 0.5 cm^2). The leaf explants were placed with the abaxial surface on the culture medium . Three to four explants were cultured in each vial containing MT basal medium supplemented with different levels of phytohormones (Appendix A).

2. 3. 4. Root explants

Tap oot segments were cut 0.5 cm length and placed on MT basal medium (Appendix A) supplemented with different levels of phytohormones.

2. 4. Assessment of growth

The responses of the explants were observed every week. The percentage of the explants responded, number of shoots and/or roots regenerated per explant and the average height of shoots and lengths of roots were recorded after 8 weeks in culture.

When calli were regenerated the degree of callusing on the explants was scored using an arbitrary defined scale:

- = no callus growth.

+ = callus size 3 - 7 mm. (small)

+ + = callus size 7 - 12 mm. (medium)

+ + + = callus size > 12 mm. (large)

callus colour, G = green, WG = whitish green

2. 5. Rooting

Well developed shoots (2 - 4 cm) regenerated from explants were excised and rooted on MT basal medium with or without 1-naphthaleneacetic acid (NAA) supplements. After the root primordia were initiated, plantlets were transferred to the MS basal medium (Appendix A) for shoot and root elongation.

All cultures were maintained under identical culture conditions as described in 2. 1. 2.. For each treatment 10-15 explants were cultured and each experiment was repeated twice.

2. 6. Acclimatisation of tissue culture plantlets

The regenerated plantlets were acclimatised under laboratory conditions at light intensity 50 - 60 µmol m^{-2} s^{-1} and 22 - 28 ± 2 °C day and night temperature. The relative humidity was reduced gradually from 95% in the first day to normal

humidity 65 - 70% after 4 weeks when the percentage of successful plantlets were recorded. Two acclimatization procedures were employed.

Procedure **a**:

Jars containing plantlets with well developed shoots and roots were loosened for one week prior to transfer of the plantlets from culture jars. The plantlets were removed from culture jars, and after washing their roots with tap water to prevent fungal and/or bacterial contamination, plantlets were transferred to 13 cm pots containing sand : soil (1:1). As relative humidity was not controlled in the laboratory in which the plantlets were maintained, the plantlets were covered with transparent plastic bags to ensure high relative humidity. Humidity was reduced by punching holes on the plastic bags gradually. After 4 weeks, the plastic bags were completely removed and plants were ready for transfer to the green house (Plate 13**a, b**).

Procedure **b**:

Similar method was employed as in procedure (**a**) but the jars were not loosened prior to plantlets transfer to soil (Plate 13 b).

2. 7. Establishment and maintenance of embryo callus

Seeds of citrumelo were sterilized as in (2. 1. 1). Cotyledons were removed in aseptic conditions and the embryos were cultured on MT basal medium (Appendix A) supplemented with 2,4-D and BAP at 2.0 and 5.0 mg/l respectively. The initiated

calli were subcultured every 4 to 6 weeks in the same media. The callus cultures were maintained in the dark at 26 ± 1 °C.

2. 8. Protoplast isolation and culture

The methods described for protoplast isolation and culture in Section (2. 8. 3) are applicable for embryo callus protoplast of citrumelo, and the mesophyll protoplasts of citrumelo, *C. suhuiensis* and *C. limonia.*

2.8.1. Enzymes for protoplast isolation

The following commercial enzymes were used for protoplast isolation and the mixtures are described in (Appendix D).

1-Cellulases

a- Cellulase R-10 (Yakult Honcha Co., Tokyo, Japan)

The enzyme was extracted from *Trichoderma viride* showing a hemicellulase activity with an optimum activity between pH 4.0 - 5.0 .

b- Driselase (Kyowa Hakko Kogyo, Tokyo, Japan)

The enzyme was obtained from *Irpex lacteus* showing a hemicellulase activity with an optimum activity between pH 5.0 and 6.0 .

2- Pectinases

a-Macerozyme R-10 (Yakult Honcha Co., Tokyo, Japan)

The enzyme was extracted from *Rhizopus arrhizus* which has a high pectinase activity within an optimum activity between pH 5.0-6.0 .

2. 8. 1. 1. Preparation of enzymes

The enzymes were dissolved in CPW salts (Appendix C) (Frearson *et al*, 1973) with 13 % mannitol and made up to volume with distilled water. The pH was adjusted to 5.8 with 1.0 M NaOH or 0.1 M HCl and the enzyme solution was filter sterilized using Millex-GU Millipore disposable filters with 0.2 µm pore size (Appendix B). The enzymes were dispensed into centrifuge tubes (10 ml) and stored frozen until required.

2. 8. 2. Isolation of protoplasts

2. 8. 2. 1. Embryo callus

One g of 8 to 10 days old calli that have prepared as in Section 2. 7. was cut into small pieces and transferred to a petri dish containing 10 ml of the enzyme mixture (Appendix D). The callus was incubated for 16 h on a rotary shaker at 45 cycles per minute at (26 ± 1 °C) in the dark. After incubation, protoplasts were isolated as in Section 2. 8. 3. .

2. 8. 2. 2. Leaf explants

Leaves were harvested from two month old aseptically cultured seedlings of citrumelo, *C. suhuiensis* and *C. limonia*, and cut into 2 mm wide strips used for protoplasts isolation. For each isolation, about 1g of leaves were cut into 1 to 2 mm

wide strips and pretreated with CPW salts (Appendix C) containing 13 % (w/v) mannitol (CPW 13M) for an hour before incubated in 10 ml of the enzyme mixture (Appendix D). Incubation was done at 26 ± 1 °C in the dark on a rotary shaker at 45 cycles per minute for 16 hours. Protoplasts were collected and washed as described in Section 2. 8. 3. (Plate 17 a).

2. 8. 3. Isolation procedures

The crude protoplasts obtained from embryo callus or leaf mesophyll were sieved through a nylon sieve (45 µm pore size) to remove the undigested tissues and cell debris. **The protoplast were suspended in CPW13M and transfered to a 16 ml sterile screw cap tubes and centrifuged at 200 x g for 5 minutes. The supernatant which contained enzymes, broken cells and debris was removed by a Pasteur pipette**. The protoplasts were resuspended in 2 ml of CPW13M and slowly added to the top of 10 ml of CPW salts containing 21% (w/v) sucrose (CPW21S) (Appendix C). After centrifuge at 250 × g for 10 minutes, a band of protoplasts appeared at the junction of the CPW21S layer and CPW13M layer. The CPW13M layer was slowly pipetted off and the protoplast band was transferred to a new centrifuge tube. This was later resuspended in CPW13M and centrifuged as before. The protoplast pellets were suspended in 2 ml of culture medium and counted with a double chamber haemocytometer under an inverted microscope to determine the yield. The protoplast suspension was later diluted to the desired density for culture.

2. 8. 3. 1. Protoplast viability test

Fluorescein diacetate (FDA) was used to determine the viability of protoplasts(Larkin, 1976). FDA stock solution was prepared at a concentration of 0.5% in acetone and stored in the freezer. To test the viability of protoplasts, a final concentration of 0.1% FDA solution was used. Two to three drops from the FDA stock solution were added to 10 ml of CPW13M solution. Equal volumes of protoplast suspension and FDA solution were mixed on a slide. After about 5 min., the protoplasts were examined under a UV light microscope and only viable protoplasts will fluoresce.

The percentage of protoplast viability was expressed as the number of fluorescing protoplasts under a UV light over the total number of protoplasts observed under light microscope in the same field. Ten to fifteen random samples were counted from 2 to 3 slide preparations (Plate 17 b).

$$\% \text{ protoplast viability} = \frac{\text{number of fluorescing protoplasts}}{\text{total number of protoplasts}} \times 100\ \%$$

2. 8. 3. 2. Protoplast diameter

It was important to measure the protoplasts diameter, to determine the appropriate sieves to be used during the protoplast isolation to maximize yield and to decide on the appropriate techniques for fusion methods. Protoplasts of uniform size will fuse better using electrical rather than chemical methods.

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Protoplast diameter was measured using gradual eye lens microscope calibrated with stage micrometer, at a magnification of 400, the scale being 2.5 μm for one division.

2. 8. 4. Methods of culturing the protoplasts .

2. 8. 4. 1. Culture in liquid media

Protoplasts isolated from the citrumelo embryo callus and from leaf explants were suspended in 4 ml of liquid culture media at a density of 1×10^5 protoplasts/ml, and placed in 5 cm plastic petri dishes sealed with Nescofilm to reduce dehydration.

2. 8. 4. 2. Culture in solid media .

The protoplast suspension at double the required final density was gently mixed with an equal volume of agarose medium kept molten at about 40 °C . Agarose was used as a gelling agent because it is has a low gelling temperature (< 30 °C) and contain low toxic substances compared to agar.

2. 8. 4. 3. Agarose droplets culture

Protoplasts were added to a mixture of agarose and culture media (1: 1). The protoplasts were cultured in droplets (15 to 20 drops per petri dish) in 5 cm petri dishes. After the protoplast and agarose mixture had solidified, the culture dishes was flooded with 3 to 4 ml of the culture medium.

2. 8. 5. Culture conditions

All the protoplast dishes were kept in the growth chamber in the dark ~~conditions~~ at 26 °C.

2. 9. Leaf Anatomy

Regenerated plantlets were taken from culture and hardened as in 2. 6. . Leaf samples were collected from plantlets in ~~culture~~, 2 weeks and 4 weeks after transplanting from culture (acclimatized plantlets). Samples from seedlings grown under laboratory conditions ~~also~~, were also taken for comparison (Plate 14, 15).

Leaf pieces from the mid region of the lamina were cut by razor blade and fixed for 48 hours in FAA solution (70% v/v ethanol, glacial acetic acid, and formalin in an 18 : 1 : 1 v/v mixture) (90 ml of 70 % alcohol, 5 ml of glacial acetic acid and 5 ml of formalin) (Appendix E). It was then transferred through a series of ethanol : tertiary butayl alcohol (TBA) (Appendix E) for dehydration and embedded in paraffin wax (milting point 55 °C). Microtome sections were prepared at 8 µm ~~thickens~~ sections. Preparations were stained with Safranine and Fast Green (Appendix E). The samples were mounted on the slides and dried at 40 °C for 4 days. The leaf sections were examined under light microscope.

2. 10. Isoenzyme

2.10. 1. Preparation of extraction

Young healthy leaves of seedlings obtained from seeds and tissue culture were harvested and used for isoenzyme analysis. The tissue culture seedlings were regenerated from internodal, leaf and root explants for citrumelo, internodal explants for *C. limonia* and from root explants for *C. reticulata*. Leaves were first washed with tap water, followed by distilled water, 1g of leaves were cut into small pieces and ground with mortar and pestle at 4 ^{o}C with 2.5 ml extraction buffer (Appendix F) to dissolve soluble protein. The homogenate was centrifuged at 10,000 rpm for 10 min.. The supernatant was collected and recentrifuged similarly as before. The clear supernatant was kept frozen for later use. All operations were carried out at 0 - 4 ^{o}C.

2. 10. 2. Preparation of gel and electrophoresis

A 7.1 % polyacrylamide gel was used (Appendix F). A vertical slab gel electrophoresis apparatus (Dual cooled vertical slab Hoofer Scientific Instruments) with a simple cooling system, was used. Glassware were washed with Decon 90 solution and rinsed with distilled water. When the gel had polymerised, 15 - 20 µl of leaf extraction were located in each well. Two gels, each containing 15 wells were normally run simultaneously. Tracking dye 1.0% (w/v) bromophenol blue was used as an indicator, which was filled at both ends of the vertical slab gel. The wells were then topped up with top buffer (Appendix F) . The tank was then fixed over the

vertical gel plates and filled with top buffer solution (which was prepared fresh at each time). The bottom tank was filled with electrode buffer solution. The cooling system was fitted into the electrophoratic tank. Electrophoresis was conducted with constant current of 15 mA and voltage 120 V at 10-15 ^{o}C for 4 - 6 h. or until the tracking dye traveled within 1 cm of the anodic end of the gel.

2. 10. 3. Isoenzyme staining

The gels was separated from the plates and isoenzyme banding patterns were identified by incubating the gels in different staining solution for 30 min. or as described in the (Appendix F) to allow the development of the bands. The reaction was stopped by rinsing the gel with tap water followed by distilled water. Gels were sliced and stained for isoenzymes malate dehydrogenase, glutamate oxaloacetate transminase, and peroxidase isoenzyem systems. All staining was carried out in the dark. Incubation was continued for 30 minutes to allow the development of the bands. Then the gels were washed with distilled water and fixed with 8% acetic acid for 12 h. to preserve the coloured reaction product, localizing the position of isoenzyme. The gels were washed with distilled water to remove shrinkage caused by fixation and stored in the fridge prior to recording of results.

2. 11. Carbon dioxide assimilation measurements

A portable LCA-4 infra-red gas analyser was used for measuring the photosynthetic rates in the largest expanded leaf of each plant. Apart from the

photosynthetic rate the LCA-4 also determined the leaf surface temperature ^{o}C, transpiration rate, and stomatal conductance.

About 6.25 cm^2 of leaf area was enclosed in the LCA-4 leaf chamber and subjected to cool white light of different intensities using a slide projector with a 200W lamp. The light intensity was varied from 10 to 1800 μmol m^{-2} s^{-1} by varying the distance between the light source and the leaf chamber. A minimum of 10 readings were taken at each point (of a particular light intensity) and CO_2 assimilation at light saturation determined each time for citrumelo, *C. limonia* and *C. reticulata*.