MATERIALS AND METHODS

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant materials

Leaf samples from the five species studied were used at different stages of growth depending on the requirements of the experiment being carried out. Leaf samples of *Citrus madurensis, C. hystrix, C. micrantha* var. *microcarpa*, citrumello (*Citrus paradisi* X *Poncirus trifoliata*) and *C. halimii* were collected from Rimba Ilmu University of Malaya, Rumah Hijau Universiti Kebangsaan Malaysia, MARDI Cameron Highlands and hill forest in Cameron Highlands respectively unless mentioned. Leaves were kept in ice prior to use.

2.1.1 Essential oils analysis

Fresh leaf samples of species mentioned above were used. Physiological age was not critical in this experiment.

2.1.2 Isoenzymes analysis

Mature, healthy leaf samples, approximately in the same physiological age were chosen. Samples were collected from plants replanted at Rimba Ilmu University of Malaya at vegetative state (not flowering) as the plants were physiologically stable and as such the banding paterns would be consistent.

2.1.3 Analysis of browning of young shoot extracts

Young growing shoots were used in this experiment. Shoots were collected from plants replanted at Rimba Ilmu University of Malaya.

2.1.4 Tissue culture experiments

Seeds of *Citrus madurensis*, *Citrus hystrix*, *Citrus micrantha* var. *microcarpa* and citrumello were used. However *Citrus halimii* seeds were not available for the experiments.

2.2 Methods

2.2.1 Extraction of the essential oils

2.2.1.1 Preparation of essential oil extracts

Fresh leaves (500 g) were first washed with tap water followed by distilled water. The clean leaves were homogenised using Wareing blender and then subjected to steam distillation for 12 hours. The distillates composing two distinguishable layers were collected. The upper layer which was slightly yellowish in colour was comprised of essential oils whereas the lower colourless layer was water. The distillates were shaken with 3 portions of diethyl ether. The water layer was removed using a separating funnel and the fraction containing the essential oils was concentrated at room temperature with a rotary evaporator (Buchi Rotavapor, model R110). Constituents of these essential oils were characterized by GCMS analysis and comparison of data was done by comparing with NBS library data basis.

2.2.1.2 Identification of the essential oil components by GCMS

GCMS analyses were performed on a Shimadzu QP-2000A mass spectrometer combined with a GC-14A under the following conditions:

A column coated with Carbowax 20 M on 60-80 Gas-Chrom Z. (length m, i.d. mm, μm film thickness) was used. The flow rate of the helium carrier gas was 1.5 ml/min. The injection volume was 1-2 μl and the split ratio was 70:1. The injector and detector temperatures were stabilized at 250°C. The following column temperature-

programming sequence was followed: initial temperature of 50°C was maintained for 2 min before being increased to 250°C at a rate of 5°C/min. The final temperature of 250°C was maintained for 30 min. The ionization energy was 70 eV with maximum temperature at 350°C.

2.2.1.3 Infrared spectrometry

All the infrared spectra were obtained by using FT-IR spectroscopy Nicolet Impact 400 taken in KBr.

2.2.2 Analysis of leaf isoenzymes

2.2.2.1 Preparation of extracts

Prior to extraction the leaves were first washed with tap water to remove excess dirt, followed by distilled water. Leaves (1 g) were cut into small pieces and ground with a glass mortar and pestle which were kept cold, with 2.5 ml extraction buffer (Appendix 1) to extract soluble protein. The homogenate was centrifuged for 10 min at 7833 *g* in a MicroCentaur (M.S.E) centrifuge. 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°C.

2.2.2.2 Preparation of gel and electrophoresis

Electrophoresis was performed using polyacrylamide gel (7.1 % (w/v)). Preparation of the gel and buffer is described in Appendix 1. A vertical slab gel electrophoresis apparatus (Dual cooled vertical slab, Hoefer Scientific Instruments) with a simple cooling system, was used in this study. Power was supplied by Atto crosspower, model 1000 Power Pack. Glasswares were washed with Decon 90 solution and rinsed with distilled water. When the gel has polymerised, 40 µl extracts were loaded 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 either at both ends of the vertical slab gel or at intervals between the sample extracts. The wells were then topped up with top buffer (Appendix 1). The top tank was then fixed to the vertical gel plate and filled with top buffer solution which was prepared fresh. The bottom tank was filled with electrode buffer solution. The cooling system was fitted into the electrophoretic tank. Electrophoresis was conducted at constant voltage of 120 V for 3-5 hours or until the tracking dye has reached the base of the gel.

2.2.2.3 Isoenzyme stainings.

The gels were separated from the plates and isoenzyme banding patterns were identified by incubating the gels in the approriate (different) staining solutions according to protocols described in Appendix 1. The reaction was stopped by rinsing the gel with tap water followed by distilled water. The gels were stained for isoenzymes

malate dehydrogenase, glutamate oxaloacetate transaminase, esterase, peroxidase and polyphenol oxidase. Rf values of the isoenzymes were scored.

$$Rf = \frac{\text{distance migrated by band}}{\text{distance migrated by bromophenol blue}}$$

2.2.3 Experiment on browning of young shoot extracts

Methods performed in this study are according to that of Esen and Soost (1974b). All operations were carried out at 0-4°C.

2.2.3.1 Preparation of shoot extracts

The shoots were homogenized in 0.05 M phosphate buffer at pH 7.2 (Appendix 2). The ratio of fresh weight to buffer volume was 1: 3. The homogenate was centrifuged for 20 min at 27,000 *g*. The colour of the supernanant was scored for 0 hour, 24 hours and 1 week after centrifugation. The colour was scored using Methuen colour charts.

2.2.3.2 Test on inhibition of browning

The shoots were homogenized in an extraction solution for inhibition of browning (Appendix 2). 0.05 M phosphate buffer (pH 7.2) was used as a control.

Colour of supernatants was scored after 0 hour, 24 hours and 1 week after centrifugation and compared with control.

2.2.4 Experiment on tissue culture of *Citrus*

2.2.4.1 Preparation of explants

Testa of seeds were removed and surface sterilized by immersing in 48 % (v/v) chlorox (2.5 % sodium hypochlorite active ingredient) for 20 min, followed by rinsing in sterile distilled water three times. The seeds were germinated in 350 ml sterile jam jar (Appendix 3) containing MSO medium (Murashige & Skoog 1962) (Appendix 4) to enhance the growth of seedling. The one month old seedlings were used as source of explants.

2.2.4.2 Culture media

Culture media used were MSO, MS1, MS2, MS24, MS25, MS26, MS27, MS28 , MS29, and MS30 (Appendix 4). The pH of media was adjusted to 5.8 with 1 N NaOH before agar (0.8 % v/v) was added. The media was autoclaved for 20 min at 121°C and 102 kPa prior to dispensing into 350 ml jam jar, 9 mm petri dishes and, 45 X 75 mm sterile universal container. The cultures were kept in the culture room with cultural conditions as in 2.2.4.3.

2.2.4.3 Culture conditions

The temperature in the culture room was at 28°C. The light condition was provided by fluorescence light for 16 hours followed by 8 hours dark cycle at the intensity of 1500-2000 lux.