CHAPTER III
MATERIALS AND METHODS

3.1. Chemicals

1.	Agar	SERVA
2.	Ammonium sulfate	Carlo Erba
3.	Borneol	Fluka
4.	Boric acid	MERCK
5.	Calcium nitrate	Carlo Erba
6.	Calcium phosphate	Fluka
7.	*trans*-Caryophyllene	Sigma
8.	Camphor	Fluka
9.	Chitosan	Aldrich
10.	*1,4*-Cineole	Fluka
11.	Citral	Aldrich
12.	*beta*-Citronellol	Fluka
13.	Cobalt chloride	Carlo Erba
14.	Copper sulfate	UNIVAR
15.	Cumene	Fluka
16.	*para*-Cymene	Fluka
17.	Dichloromethane	MERCK
18.	Ethanol	MERCK
19.	Ethylenediamine tetraacetic acid	UNIVAR
20.	Eucalyptol	Fluka
21.	Eugenol	Fluka
22.	Ferrous sulfate	Carlo Erba
23.	Geranyl acetate	Aldrich
24.	Glycine	UNIVAR

25.	Geraniol	Fluka
26.	Hexadecane	Aldrich
27.	*alpha*-Humulene	Aldrich
28.	Hydrochloric acid	MERCK
29.	Limonene	Fluka
30.	Linalool	Fluka
31.	Magnesium sulfate	Carlo Erba
32.	Manganese sulfate	J.T. Baker
33.	Menthol	Aldrich
34.	Menthyl acetate	Fluka
35.	Myo-inositol	Sigma
36.	Nerol	Fluka
37.	Nicotinic acid	Carlo Erba
38.	Pentadecane	Aldrich
39.	*alpha*-Phellandrene	Aldrich
40.	*alpha*-Pinene	Fluka
41.	Potassium iodide	UNIVAR
42.	Potassium nitrate	Carlo Erba
43.	Potassium phosphate	Carlo Erba
44.	Pyridoxine hydrochloride	Sigma
45.	Sodium hydroxide	Mallinckrodt
46.	Sodium molybdate	Carlo Erba
47.	Sucrose	UNIVAR
48.	*gamma*-Terpinene	Fluka
49.	Terpine-*4*-ol	Fluka
50.	Thylmol	Fluka
51.	Thiamine hydrochloride	Sigma
52.	Zinc sulfate	Fluka

3.2. Instruments

1. Analytical balance
   Sartorius ME235P
2. Autoclave
   Hirayama HA-300
3. Gas chromatography/mass spectrometry
   Shimadzu GCMS QP 2010 Plus
4. Micropipet
   SOCOREX, Acura 825
5. Microwave oven
   LG MS-2127CW
6. pH meter
   Hanna Instruments HI 8417
7. Shaker
   Gerhardt RO500
8. Syringe Filters
   Millex SLGV033RS

3.3. Materials

Orchid plant samples, including *R. gigantea*, *R. gigantea* var. *harrisonianum*, *V. coerulea* and *D. parishii* were collected at Chiang Mai, Thailand in 2011 (flowering time during February-March).

All of these orchids were identified at the Queen Sirikit Botanical Garden, Ministry of Natural Resources and Environment, Thailand.

3.4. Time and place

Cell cultures were carried out at the Tissue Culture Unit, Department of Pharmacognosy, Faculty of Pharmacy, Rangsit University and volatile constituents

were analyzed at The Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University during 2009-2011.

3.5. Methods

3.5.1. Extraction and identification of essential oil from fresh plant of orchids.

Orchids, including *R. gigantea*, *R. gigantea* var. *harrisonianum*, *V. coerulea* and *D. parishii* were collected and separated into leaf, root, flower and/or pseudobulb parts. In each part, aromatic compounds of orchid samples were extracted by HS-SPME and analyzed by using gas chromatography/mass spectrometry. One hundred milligram were placed in 20 ml glass vials. The vials were tightly capped with a PTFE-silicon septum. An AOC 5000 Combi PAL SPME holder with a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber assembly was used. The fiber was preconditioned according to manufacturer. Vials containing samples were pre-equilibrated and heated at 80 ^{o}C for 30 min. on a heating platform with agitation at 500 rpm. After extraction, the fiber was desorbed into GC injector for 2 min.

The GC-MS used was a Shimudzu GCMS-QP 2010 Plus system. The column was a 30 m × 0.25 mm DB-5 MS capillary with 0.25 µm film thickness. The carrier gas was helium at a flow rate of 1 ml/min. Samples were injected by placing the SPME fiber at GC inlet for 2 min. The starting oven's temperature was 60 ^{o}C, then raised to 200 ^{o}C at a rate of 5 ^{o}C/min and held at 200 ^{o}C for 10 min. The mass spectrometry was operated in the electron impact mode with ion source temperature 200 ^{o}C, using an ionization voltage of 70 eV. The mass range was 40-400 amu. Volatile compounds were identified by comparing the obtained mass spectra of relevant chromatographic peaks with spectra of the WILEY 7 library, NIST Chemistry WebBook and Kovats retention indices with other published mass spectra[54,111].

3.5.2. Preparation of media

Modified Vacin and Went (VW, 1949) was used as a media thought out this experiment. The constituents for VW media as modified were[56]

Tricalcium phosphate	200	mg/l
Potassium nitrate	525	mg/l
Monopotassium acid phosphate	250	mg/l
Magnesium sulfate	250	mg/l
Ammonium sulfate	500	mg/l
Manganese sulfate	6.8	mg/l
Sucrose	20	g/l
Agar	8	g/l
Water	845	ml
Coconut water	150	ml
Iron chelate	5	ml

The media were prepared by the use of stock solution as follows:

Stock solution A:

Potassium nitrate	5.25	g/l
Monopotassium acid phosphate	2.50	g/l
Ammonium sulfate	5.00	g/l
Manganese sulfate	0.068	g/l

Stock solution B:

Magnesium sulfate	2.50	g/l

Approximately 200 ml of distilled water was measured and poured into a beaker. Five ml Iron chelate was added and then 100 ml stock solution A and B were added. Calcium phosphate was dissolved with 1 M hydrochloric acid. After the media completely dissolved, coconut water and sucrose were added. The pH was adjusted to 5.0 with few drops of 1 M sodium hydroxide or 1 M hydrochloric acid. The liquid media was poured into 250 ml Erlenmeyer flasks which were covered with aluminium foil and then sterilized in an autoclave at 121 ^{o}C for 15 min.

3.5.3. Aseptic work

All the manipulations were carried out under aseptic conditions using a laminar flow cabinet with horizontal flow of sterilized air.

3.5.4. Suspension culture

Protocorms were induced from seeds of *D. parishii*. Suspension cultures were initiated by transferring healthy protocorms to an Erlenmeyer flask containing 50 ml liquid media. Then the flasks were put on an orbital shaker, circulating at 120 rpm with the temperature of 25±2 ^{o}C under continuous light using cool white fluorescent tubes. The suspension cultures were subcultured to new fresh liquid media at 4 weeks interval.

3.5.5. Fresh and dry weight measurements

Increase in fresh weight can measure without sacrificing the samples at the beginning of an experiment. Transfer cells to pre-weight culture flask containing the media. Weight the flask again and determine the weight of cell added. At the end of an experiment, remove the entire cells from suspension culture and determine the final weight. After measuring the fresh weight, dry the samples in an oven at 60 ^{o}C until no change in dry weight is observed.

The statistical technique for testing the significant difference among the treatments for this experiment was calculated by one-way Analysis of Variance (ANOVA).

3.5.6. Extraction and identification of essential oil from plant cell culture of *D. parishii*

Plant cell cultures were dried for 24 hr by freeze dryer. Volatile compounds of plant cell cultures were extracted by HS-SPME and analyzed by gas chromatography/mass spectrometry in the condition above in 3.5.1.

3.5.7. Improving of essential oil level production in plant cell culture

3.5.7.1. Elicitation with chitosan

Chitosan solution was obtained by dissolving in 0.1 M acetic acid and adjusted the pH to 5.0. Chitosan concentration was varied from 50 to 400 ppm for determination of optimum concentration. Cell cultures were used over for 28 days. The control experiment was carried out by adding each concentration of chitosan to media without cells under the same conditions as the culture. The volatile components were obtained and measured by the method described in 3.5.6.

3.5.7.2. Permeabilization

Tween 20 was used as the permeabilizing agent with various concentration of 0.5, 1.0%, 1.5%, 2.0% w/v. The volatile components were obtained and measured by the method described in 3.5.6.

3.5.7.3. Two-phase system

Hexadecane was used as the secondary phase for accumulating essential oil constituents released in media. Ten ml hexadecane was added into media and then autoclaved prior to use. The volatile components were obtained and measured by the method described in 3.5.6.

3.5.7.4. Feeding precursor

Substrates were obtained commercially with the purity over 98%. They were mixed with 70% ethanol to get clear solution and added to the suspension cultures by passing through 0.22 µm membrane filter. The final concentrations of substrates were 100 ppm. The control

reading was made without substrates added to cultures and media added substrates without cell cultures. The cultures were incubated in the conditions which had mentioned above in 3.5.4. After incubation, the cultures were harvested and analyzed by the method in 3.5.6.