Chapter 3

Material and Methods

Part I Studies on factors influencing growth and *in vitro* tuberization.

This part of the studies was carried out to investigate the effects of some chemicals containing in the culture medium and a physical condition, i.e. light illumination on seedling growth and *in vitro* tuberization of *Pecteilis sagarikii* Seidenf. It was divided into five experiments as follows:

Experiment 1 Effect of sucrose concentration

This experiment was planned in Completely Randomized Design with 6 levels of sucrose concentrations, i.e. 0 1 2 3 5 and 7 % (w/v). A basal medium was CMU_1 (Appendix A). Each sucrose concentration had 20 replicates. Ten milliliters of the medium was filled in a 25x150 mm test tube and polypropylene sheet was used as the culture vessel lid.

Protocorms at pre-tuberization stage, germinated from 7-week-old seeds on the CMU_1 medium in continuous dark condition, kept at 25±1 degree Celsius (ºC) were used. The cultures were illuminated at 2.78 µmole.m^{-2}.s^{-1} for 1 week, and subsequently increased to 27.8 µmole.m^{-2}.s^{-1} for 16 hours/day until the end of the study.

Growths of seedlings were collected in terms of plant height, number of leaf and leaf size, tuber information and root information.

Experiment 2 Effect of coconut water concentration

This experiment was also planned in Completely Randomized Design with 5 levels of coconut water concentrations, i.e. 0 5 10 15 and 20 % (v/v). The basal medium was CMU_1 (Appendix A). Each coconut water concentration had 20 replicates. Ten milliliters of the medium was filled in a 25x150 mm test tube and polypropylene sheet was used as the culture vessel lid.

Protocorms at pre-tuberization stage with the same culture history as in Experiment 1 were used. The cultures were illuminated as in Experiment 1.

Data collection was the same as in Experiment 1.

Experiment 3 Effects of coconut water and BAP concentrations

This experiment was designed in factorials in Completely Randomized Design with 2 main factors: Coconut water concentration at 2 levels, i.e. 0 and 15 % (v/v), and BAP concentration at 5 levels, i.e. 0 0.1 0.5 1.0 and 2.0 mg/l. The basal medium was CMU_1 (Appendix A). Combinations of coconut water and BAP concentrations were shown in table 1. Each treatment had 10 replicates. Ten

milliliters of the medium was filled in a 25x150 mm test tube and polypropylene sheet was used as the culture vessel lid.

Table 1 Combinations of coconut water and BAP concentrations.

Coconut water (%)	BAP concentration (mg/l)				
	0	0.1	0.5	1.0	2.0
0	*T1**	*T2*	*T3*	*T4*	*T5*
15	*T6*	*T7*	*T8*	*T9*	*T10*

* *T* = treatment

Protocorms at pre-tuberization stage with the same culture history as in Experiment 1 were used. The cultures were illuminated as in Experiment 1.

Data collection was the same as in Experiment 1.

Experiment 4 Effects of IAA, BAP and coconut water concentrations in the dark condition

This experiment was planned in Completely Randomized Design with 9 treatments. The basal medium was CMU_1 without organic substances I (Appendix A), except in the control. Growth regulators used in each treatment was shown in table 2. Each treatment had 20 replicates. Ten milliliters of medium was filled in a 25x150 mm test tube and polypropylene sheet was used as the culture vessel lid.

Table 2 Combinations of growth regulators and coconut water in each treatment.

	IAA (mg/l)	BAP (mg/l)	Coconut water (%)
Control *	0	0	0
T1	0	0	0
T2	0	0	15
T3	0.01	0	0
T4	0.10	0	0
T5	1.00	0	0
T6	0	0.01	0
T7	0	0.05	0
T8	0	0.10	0

* = No growth regulator, but contained added organic substances.

Protocorms at pre-tuberization stage with the same culture history as in Experiment 1 were used. The cultures were placed in continuous dark condition at 25±1°C.

Growth parameters were recorded as in Experiment 1. After the data had been recorded, plantlets were separated into plant parts, i.e. protocorm, shoot and tuber, to be analyzed for some carbohydrate contents. Fresh samples were extracted with 80 % ethanol (Appendix C-1b) and the phenol-sulfuric acid assay (Appendix C-5) was used to determine total soluble sugar. The residues were, thereafter, extracted

with 8.14N $HClO_4$ (Appendix C-2). The anthrone method (Appendix C-6) was used to determine starch contents.

Experiment 5 Effects of illumination and sucrose concentration

This experiment was planned in factorials in Completely Randomized Design with 2 main factors: Illumination, i.e. continuous darkness and lighting at 27.8 µmole.m^{-2}.s^{-1} for 16 hours/day, and sucrose at 5 concentrations, i.e. 0 1 2 3 and 5 % (w/v). The basal medium was CMU_1 without coconut water (Appendix A).

Combinations of illumination and sucrose concentrations were shown in table 3. Each treatment had 20 replicates. Ten milliliters of the medium was filled in a 25x150 mm test tube and polypropylene sheet was used as the culture vessel lid.

Table 3 Combinations of illumination and sucrose concentrations.

Illumination	Sucrose concentration (%)				
	0	1	2	3	5
Cont. darkness	*T1*	*T2*	*T3*	*T4*	*T5*
Light 16 h/d	*T6*	*T7*	*T8*	*T9*	*T10*

Protocorms at pre-tuberization stage with the same culture history as in Experiment 1 were used. The cultures were placed in the illumination following the treatments.

Growth parameters were recorded as in Experiment 1. After the data had been recorded, plantlets were separated into plant parts, to be analyzed for both the total soluble sugar and the starch contents with the same methods as in Experiment 4.

Part II Histological study on tuberization.

The aim of this part was to investigate histological changes during tuberization of *Pecteilis sagarikii* Seidenf. The study was carried out in 2 phases of life cycle.

1. The first tuberization of a seedling

The study in this phase was on *in vitro* first tuberization from the seedlings derived from protocorms of germinated seeds.

Seven-week-old seeds after pollination were sown in aseptic condition on the CMU_1 medium. The culture vessels were placed under continuous darkness at 25±1°C. Various stages of protocorms and seedlings were collected and prepared for histological study using paraffin embedding technique (Appendix B-1).

The biggest-size samples that could not be used in the paraffin embedding technique were longitudinal cut into very thin sections using freezing-microtome with an appropriate new modified technique (Appendix B-2).

Series of tissue sections were observed under light microscope with 40X and 100X magnifications to follow the changing of tissues during tuberization.

2. The tuberization of a mature plant

This study on tuberization of mature plant was carried out in greenhouse-grown plants. The plants grew from early sprouting tubers (in April) in 5 inches plastic pots using mixed growing media, i.e. coarse sand : rice husk charcoal : loam at a ratio 3:2:1 by volume. Different stages of plant were observed, collected and then prepared for histological study by using paraffin embedding technique (Appendix B-1) and/or using freezing-microtome with the new modified technique (Appendix B-2).

Series of tissue sections were observed under light microscope to follow the changing of tissues during tuberization.

Part III Studies on the changing of internal macro elements, free amino acids and some growth regulators in mature plants during tuberization.

The studies in this part were carried out to investigate changing of some internal chemicals during tuberization in *Pecteilis sagarikii* Seidenf. mature plant growing in a greenhouse. Four stages of plant were determined as follows:

Stage 1 Dormancy stage
Stage 2 Early sprouting stage
Stage 3 Vegetative stage
Stage 4 Pre-flowering stage

Each stage has 4 replicates with 6 randomized plants per replication. Plant samples were divided into three parts, i.e. shoot, tuber and root. They were washed in running water and rinsed with de-ionize distilled water, cut into small pieces and then dried using a freeze dryer. The freeze-dried samples were ground with sample mill and kept in paper bags under an ultra low temperature at -20°C.

The samples were used in the following analyses.

1. Analysis of macro elements

1.1 Analysis of total nitrogen contents

The sample powder was digested using the modified Kjeldahl method (Appendix C-3) and determined total nitrogen contents by using the indophenol method (Appendix C-7).

1.2 Analysis of phosphate contents

The sample powder was digested using the modified Kjeldahl method (Appendix C-3) and determined phosphate contents by using the ammonium molybdate method (Appendix C-8).

1.3 Analysis of potassium, calcium and magnesium contents

The sample powder was digested using the $HClO_4$-HNO_3 method (Appendix C-4) and determined K, Ca and Mg contents by using atomic absorption spectrophotometer (Hitachi model Z-8200 Series Polarized Zeeman) at wavelength 766.5, 422.7 and 285.2 nm, respectively (Appendix C-9).

2. Analysis of free amino acid contents

The sample powder was extracted with 80 % ethanol (Appendix C-1a) and measured total amino acid contents using spectrophotometer at 570 nm. The extracted solutions were subsequently diluted until having the total amino acid about 50-500 µM before determining free amino acids by using High Performance Liquid Chromatography (HPLC) (Appendix C-10).

3. Analysis of indoleacetic acid (IAA) and abscisic acid (ABA) contents

The sample powder was extracted, and purified following the protocol that modified by Associate Professor Dr. Ohtake Norikuni (Appendix C-11). Gas Chromatography Mass Spectrophotometer (GCMS) were used to measure both IAA and ABA contents by comparative measuring with standard radioisotope, i.e. ^{13}C-IAA and ^{2}H-ABA.