Chapter 3

Materials and Methods

1. Plant Materials

Mature seeds of *J. curcas* L. or Physic nut were harvested from Mae Hia Agricultural Research and Training Centre, Faculty of Agriculture, Chiang Mai University, in August 2009.

2. Methods

Part 1 Studies on callus induction from endosperm explants of Physic nut (*J. curcas* L.)

The part of the studies was carried out to find out a formulation of medium that could be used to induce callus from the endosperm of *J. curcas* L.

Experiment 1 Effects of auxin types and concentrations on callus induction from endosperm explants of J. curcas L.

This experiment was planned in 3x3 factorial in completely randomized design with 3 types of auxin, i.e. 2,4-D, NAA and IBA and 3levels of auxin concentrations, i.e.0, 5 and 10 micromolar (μM). Combinations of auxin types and concentrations were shown in table 6.A total of thirty explants were cultured in each treatment with three replications. Details are as follows

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1.1 Seeds sterilization and initiation

Mature seeds of physic nut were washed under running tap water for 15 minutes to remove soils and other contaminants on the outer surface of seed coats. After that, the seeds were thoroughly rinsed in 70% (v/v) ethanol for 1 minute and then were washed three times with sterilized distilled water. Additionally, the seeds were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 minutes and they were washed three times in sterilized distilled water. After that, the seeds were immersed in

95% ethanol and they were briefly flamed. Endosperm from seeds was cut to size of 0.5x0.5 cm.

1.2 Media and culture conditions

Medium used to induce callus from these explants was Murashige and Skoog (MS) medium (1962) (Appendix A), 3% (w/v) of sucrose and 0.8% (w/v) agar were added. The pH was adjusted to 5.6±0.02 using a pH meter prior to autoclave at 121°C for 15 min. All culture incubated at 25±1°C, under darkness condition.

1.3 Effects of growth regulators on callus induction

Callus induction studies were carried out by culturing the sterilized endosperm explants in MS media containing 2,4-D, NAA and IBA at concentrations of 5 and 10 µM (showed in table 6). The control medium was MS, without any addition of plant growth regulators. A total of thirty explants were cultured in each treatment with three replications. The percentage of explants forming callus, degree of callogenesis and morphologies (callus texture, callus color) were observed in one month

Table 6 Auxin types and concentrations

Auxin types	**Auxin concentrations (µM)**		
	0	**5**	**10**
2,4 - D	T1	T2	T3
NAA	T4	T5	T6
IBA	T7	T8	T9

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Each experiment was set up under a completely randomized design. Data were subjected to one-way ANOVA. Differences among means were tested for significance by Turkey HSD all-pairwise comparison test at 0.05 level of probability.

Part 2 Study on growth and oil content from cell suspension culture of endosperm cell of Physic nut (*J. curcas* L.)

The part of the studies was carried out to find out the formulation of medium and optimum condition that appropriate for growth and oil production from jatropha endosperm suspended cells *in vitro*. It was divided into three experiments as follows

Experiment 1 Growth and oil content from cell suspension culture of endosperm cell of J. curcas L.

This experiment was planned from the selected condition of Part I in a completely randomized design with three replications to study the possibility to produce oil from endosperm suspended cells *in vitro*. Details are as follows

1.1 Cell suspension culture

Cell suspension cultures were initiated by inoculating 1 gram of fresh friable callus into 30 ml of MS liquid media. Medium for suspension culture was supplemented with 10 μM/L NAA. Cultures were incubated continuously on a rotary shaker (120 rpm) under dark condition at 25±2°C for 40 days.

1.2 Growth measurement and analysis of oil content

Number of cells, packed cell volume (PCV), fresh weight (FW) and dry weight (DW) of cells were measured as growth. Oil production of suspended cells was measured and expressed in terms of total lipids extract (TLE). Total lipid extract analysis was conducted following the method from Zou *et al*. (1995) who described as follows; the cells were mixed with 1 ml isopropanol, followed by sonication for 10 minutes. The mixtures were then heated in boiling water for 5 minutes. The solution was immediately cooled, prior to addition of 0.5 ml of CH_2Cl_2.The mixture was set aside for 30 minutes at room temperature, and shaked occasionally using a vortex mixer. The organic and aqueous phases were then separated by the sequential addition of 2 ml of CH_2Cl_2 and of 2 ml of 1 M KCl in 0.2 MH_3P0_4. After the sample was centrifuged (x500g) for 5 minutes, the lower organic phase was collected and the aqueous phase was washed twice with 2 ml of CH_2Cl_2. The original organic phase was then mixed with the washes and dried to yield the total lipid extract (TLE). The growth of suspended cell and TLE analysis were measured every 5 days until 40 days. All parameters were repeated three times.

Each experiment was set up under a completely randomized design (CRD) with three replicates. Data were subjected to one-way ANOVA. Differences among means were tested for significance by Turkey HSD all-pairwise comparison test at 0.05 level of probability.

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Experiment 2 Effects of callus amount on growth and oil contents of endosperm cell of J. curcas L.

The objective of this study was to find out the effects of callus contents on growth and oil contents of endosperm cell of *J. curcas* L. The experiment consisted of 3 treatments; 1 gram, 2 grams and 3grams of callus amount and used a completely randomized design (CRD) for experimental design. Friable calli of *J. curcas* L. were cultured in liquid MS medium supplemented 10µM 1-Naphthaleneacetic (NAA) and with 30g/L sucrose. The cultures were placed on 120rpm rotary shaker and kept under darkness at 25±2°C for 20days.The growth consist of fresh weight (FW), dry weight (DW), the number of cells, packed cell volume (PCV) and oil content (%w/w) of each treatments were measured. Details are as follows

2.1 Cell suspension culture

Cell suspension cultures were initiated by inoculating 1 gram of fresh friable callus into 30 ml of MS liquid media. Medium for suspension culture was supplemented with 10 µM NAA. Cultures were incubated continuously on a rotary shaker (120 rpm) under dark condition at 25±2 ºC.

2.2 Growth measurement and analysis of oil content

Number of cells, packed cell volume (PCV), fresh weight (FW) and dry weight (DW) of cells were measured as growth. Oil production of suspended cells was measured and expressed in terms of total lipids extract (TLE). Total lipid extract analysis was conducted using the method from Zou *et al*. (1995) who described as follows; the cells were mixed with 1 ml isopropanol, followed by sonication for 10 minutes. The mixtures were then heated in boiling water for 5 minutes. The solution was immediately cooled, prior to addition of 0.5 ml of CH_2Cl_2. The mixture was set aside for 30 minutes at room temperature, and shaked occasionally using a vortex mixer. The organic and aqueous phases were then separated by the sequential addition of 2 ml of CH_2Cl_2 and of 2 ml of 1 M KCl in 0.2 MH_3P0_4. After the sample was centrifuged (x500g) for 5 minutes, the lower organic phase was collected and the aqueous phase was washed twice with 2 ml of CH_2Cl_2. The original organic phase was then mixed with the washes and dried to yield the TLE. The growth of suspended cell and TLE analysis were measured every 5 days until 20 days were same measures.

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Each experiment was set up under a completely randomized design (CRD)for experimental design with three replicates. Differences among means were tested for significance by Turkey HSD all-pairwise comparison test at 0.05 level of probability.

Experiment 3 Effects of low temperature and sucrose concentrations on growth and oil contents of endosperm cell of J. curcas L.

The experiment has two main factors consisted temperature at 3 levels include 15, 20, 25 degree Celsius and 5 concentrations of sucrose include 20, 25, 30, 35 and 40 gram per litter (Table 7). It was conducted in a 3x5 factorials in completely randomized design (CRD)with three replications. Two grams of friable calli of *J. curcas* L. were cultured in liquid MS medium supplemented 10 µM 1-Naphthaleneacetic (NAA). The cultures were placed on 120 rpm rotary shaker and kept under darkness for 20 days. The growth consist of fresh weight (FW), dry weight (DW), the number of cells, packed cell volume (PCV) and oil content (%w/w) of each treatments were measured.

Table 7 Combinations of temperature levels and sucrose concentrations.

Temperature (°C)	Sucrose concentrations (g/l)				
	20	25	30	35	40
15	T1	T2	T3	T4	T5
20	T6	T7	T8	T9	T10
25	T11	T12	T13	T14	T15

3.1 Cell suspension culture

Cell *1*

suspension cultures were initiated by inoculating 1 gram of fresh friable callus into 30 ml of MS liquid media. Medium for suspension culture was supplemented with 10 µM NAA. Cultures were incubated continuously on a rotary shaker (120 rpm) under dark condition at 25±2 ºC.

3.2 Growth measurement and analysis of oil content

Number of cells, packed cell volume (PCV), fresh weight (FW) and dry weight (DW) of cells were measured as growth. Oil production of suspended cells was measured and expressed in terms of total lipids extract (TLE). Total lipid extract

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analysis was conducted using the method from Zou *et al.* (1995) who described as follows; the cells were mixed with 1 ml isopropanol, followed by sonication for 10 minutes. The mixtures were then heated in boiling water for 5 minutes. The solution was immediately cooled, prior to addition of 0.5 ml of CH_2Cl_2.The mixture was set aside for 30 minutes at room temperature, and shaked occasionally using a vortex mixer. The organic and aqueous phases were then separated by the sequential addition of 2 ml of CH_2Cl_2 and of 2 ml of 1 M KCl in 0.2 MH_3P0_4. After the sample was centrifuged (x500g) for 5 minutes, the lower organic phase was collected and the aqueous phase was washed twice with 2 ml of CH_2Cl_2. The original organic phase was then mixed with the washes and dried to yield the TLE. The growth of suspended cell and TLE analysis were measured every 5 days until 15 days, were same measures.

Each experiment was set up under a 3x5 factorials in completely randomized design for experimental design with three replicates. Data were subjected to one-way ANOVA. Differences among means were tested for significance by Turkey HSD all-pairwise comparison test at 0.05 level of probability.

Part 3 Study on oil production from endosperm cells of Physic Nut (*J. curcas* L.) in a modified bubble column bioreactor.

The study of the producing oil from the endosperm cells of *J. curcas* L. was conducted in 1 of liter modified bubble column bioreactor, contained liquid MS medium supplemented with 10µM 1-Naphthaleneacetic acid (NAA) alone and 35 g/L of sucrose, pH 5.6±0.2. The culture was incubated under dark condition, at 20±3 ℃ and 0.3 L/min of air flow rate for 20 days. The growth and oil contents were evaluated at the last day of the cultured with three replications. Details are as follows

3.1 In vitro vegetable oil production in a modified bubble column bioreactor

A total of 40 g of endosperm callus was introduced into 600 ml modified MS medium which is selected from experiment 2 data. Culture was incubated in a modified bubble column bioreactor treated with low temperature levels (20 degree Celsius selected from experimental data in experiment 2.3), with air flow rate 0.2 L/min under dark condition for 15 days.

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3.2 Growth measurement and analysis of oil content

Number of cells, packed cell volume (PCV), fresh weight (FW) and dry weight (DW) of cells were measured as growth. Oil production of suspended cells was measured and expressed in terms of total lipids extract (TLE). Total lipid extract analysis was conducted using the method from Zou *et al.* (1995).Once separated from the medium, endosperm suspended cell that has been maintained in the bioreactor for 15 days was sonicated for 10 minutes. Samples were dried at 80° until weight was stable, then weighed as much as 0.1 g and then placed on the tube. It was added with 1 ml of isopropanol, and then heated in boiling water for 5 minutes. After chilling, the sample was added with 0.5 ml of CH_2Cl_2 and left for 30 minutes while shaking occasionally. Organic phase was separated from the water phase by adding 2 ml of CH_2Cl_2 and 2 ml of KCl solution in 0.2 M H_3PO_4. The samples were centrifuged at 500 g for 5 minutes. Organic phase on the bottom layer were stored, while its water phase was washed twice with CH_2Cl_2. Organic phase obtained was combined, then dried and weighed to obtain total lipid extracts. The growth of suspended cell and TLE analysis were measured at 20 days. Characteristic of culture, i.e. color, pH were collected.

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