CHAPTER 3
METERIALS AND METHODS

3.1 Meterials, Chemical reagents and Equipments

A. Plant meterial

Plant meterial use in this experiment is Plookao (*Houttuynia cordata* Thunb.) plants. The plants were collected from Banhouysai, Maehea subdistrict, Chiang Mai province, Thailand. The leaf, stem and rhizome segments from 4 to 5 month-old Plookao (*Houttuynia cordata* Thunb.) were used as explants sources. Three types of explants including the second leaf from above, 3-4 node stem from the ground and underground stoloniferous rhizomes.

B. Chemical reagents

- Sterilized Chemical

Name of chemical reagent	**Product company**
NaOCl (Sodium hypochloride)	Merck Co.
$HgCl_2$ (Mercuric chloride)	POCh S.A. Co.
70% ethanol	Vetchawit
95% ethanol	Vetchawit
Tween-20®	Vetchawit

- **Plant tissue culture medium**

Culture medium used in this experiment is MS (Murashige and Skoog, 1962). The chemicals used for media preparation are as follow

Name of chemical reagent	**Product company**
NH_4NO_3	S.K. Trading
KNO_3	Merck
$CaCl_2.2H_2O$	Merck
$MgSO_4.7H_2O$	Sigma
KH_2PO_4	Merck
$FeSO_4.7H_2O$	Sigma
Na_2EDTA	Ajax
H_3BO_3	Sigma
$MnSO_4.4H_2O$	Merck
$ZnSO_4.4H_2O$	Merck
KI	Sigma
$Na_2MoO_4.2H_2O$	Sigma
$CuSO_4.5H_2O$	Sigma
$CoCl_2.6H_2O$	Sigma
Glycine	Merck
Nicotinic acid	Sigma
Pyridoxine-HCL	Fluka
Thiamine-HCL	Fluka
Agar	Himedia

- **Buffers for pH meter**

Name of chemical reagent	**Product company**
HCl 1 N	J.P.DAKER, USA
NaOH 1 N	Vetchawit

- Plant Growth Regulators

Name of chemical reagent	Product company
Auxins	
2,4-Dichlorophenoxyacetic acid (2,4-D)	Fluka, Switzerland
Indole-3-butyric acid (IBA)	Fluka, Switzerland
Cytokinins	
6-Benzyl-aminopurine (BAP)	Fluka, Switzerland

C. Equipments

1. Alcohol lamp
2. Culture vessels: wide necked bottle with fitted lids sized 8 onz.
3. Handling pipette and sterile pipette tip
4. Weighing balance 1 to 200 g
5. Weighing balance 1.0-0001 g
6. pH-meter
7. Hot plate with magnetic stirrer
8. Autoclave
9. Sterilized forceps (18 cm long)
10. Sterilized scalpels (15 cm long) and blades
11. Laminar flow cabinet
12. Glassware-Duran bottles with rims, glass cylinders, beakers
13. Glass rod stirrers
14. Sterilized petri dishes
15. Gyratory shaker
16. Sterilized rack (autoclaved)
17. Plastic syringes sized 20, 60 cc.
18. Hot air oven for drying glassware
19. Hot air oven for drying sample
20. Microwave oven
21. Paper towel

22. Trolley for carrying hot media flasks and containers
23. Aluminum foil
24. Refrigerator
25. Sterilized distilled water (autoclaved)
26. Cool white fluorescent light source
27. Spray bottle
28. Duran bottles, glass cylinders, beakers sterrers
29. Micropipette (20 µl, 200 µl, 1,000 µl and 5 ml) and pipette tip
30. GC-MS analysis machines from Agilent Technologies 5973inert Mass Selective Detector
31. Watman filter No. 1
32. Evaporator
33. Blender
34. Sample tube
35. Volumetric flask

3.2 Medium preparation

The culture media used for this study were ½MS and MS medium (see on Appendix A). The explants sterilization used MS basal medium without plant Growth regulators. Shoot multiplication and callus induction were conducted on ½MS and MS supplemented with different concentrations and combination of Auxin and cytokinins. For induction of adventitious roots, shoot clusters were subculture in ¼MS, ½MS, MS and MS supplemented with indole-3-butyric acid (IBA) at 20.00 µM. The composition of MS medium is shown in appendix A.

3.3 Methods

3.3.1 Explants Sterilization

A. Type of chemical

The chemical reagent used in this experiment were sodium hypochloride (NaOCl) and mercuric chloride ($HgCl_2$) with different concentration that are NaOCl at 2%, 4% and 6% (v/v) and $HgCl_2$ at 0.1%, 0.3%, 0.5% (w/v).

B. Time

Explants were sterilized with each chemical reagent in Table 3.1 for 5 and 10 minutes before incubation.

C. Explant types

Three types of explants including leaf, stem and rhizome used in this experiment were subjected to sterization condition that shown in Table 3.1 and Figure 3.1. After that, explants were cut and placed on MS media, and incubation at 25 ^{o}C for 16 hours in light and 8 hours in dark. Finally, explants were observed for contamination and regeneration. There were 10 bottles (3 sample each) for each treatment with three explants in each bottle and each treatment was replicated 5 times, (n = 150).

Table 3.1 Experimental set for explants sterilization

Chemicals	Concentration (%)	Time (Min.)	Explant Types
NaOCl (Sodium hypochloride)	2 4 6	5 and 10	Leaf Stem Rhizome
$HgCl_2$ (Mercuric chloride)	0.1 0.3 0.5		

- Statistical analyses

The contamination and regeneration experiments were conducted using a completely randomized design (CRD). Data present in the form of percentage. The

mean value of treatment were compared using the Turkey's Studentized Range (HSD) Test at p less than 0.05.

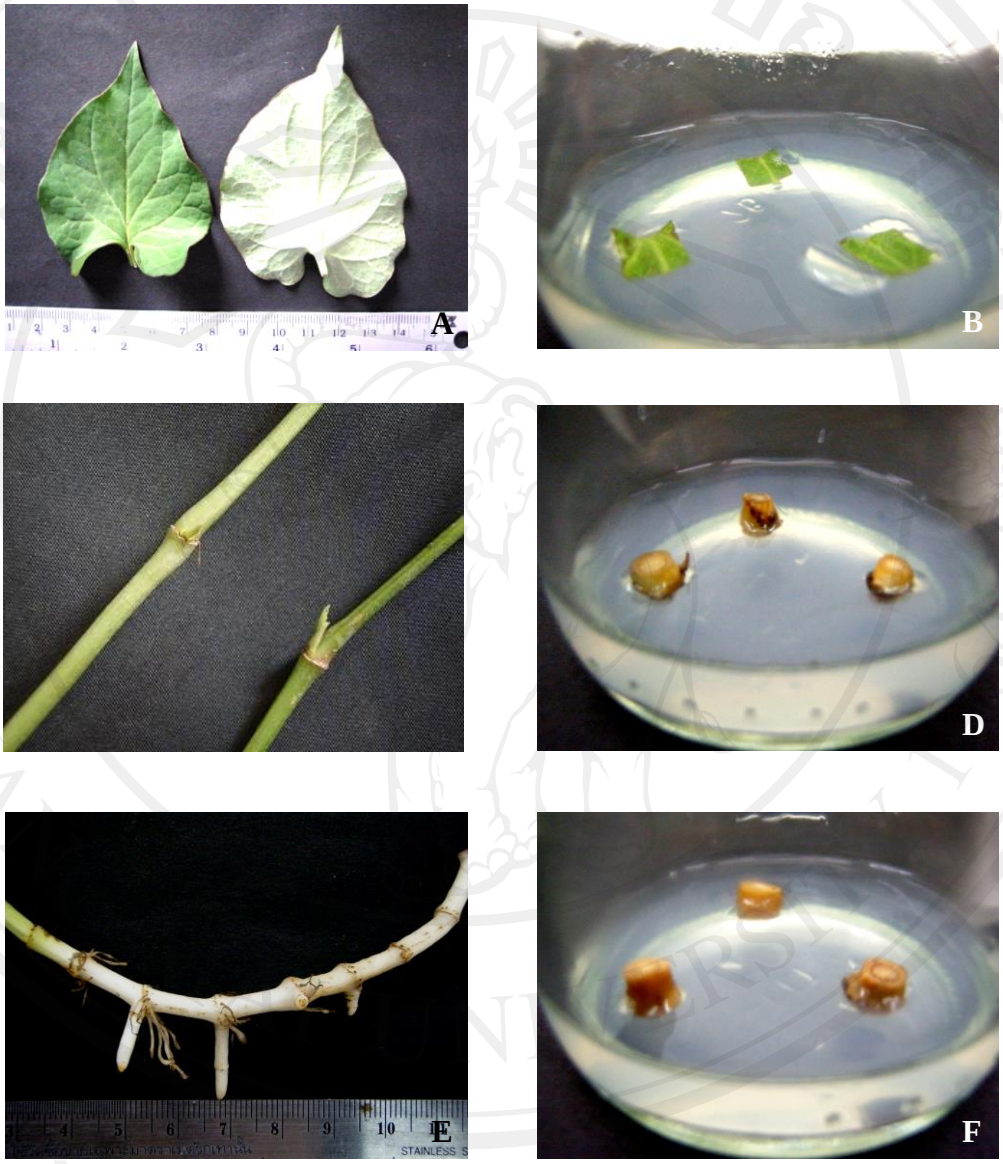

Figure 3.1 Types of Plookao explants used for *in vitro* regeneration.

A) Plookao's leaves
B) Leaves explants cultural in MS media
C) Plookao's stem
D) Stem explants cultural in MS media
E) Plookao's Rhizome
F) Rhizome explants culture in MS media

3.3.2 Shoot multiplication and callus induction

The combined effect of four Auxins (2,4-D) concentrations and four cytokinins (BAP) concentrations for the regeneration of stem (The result from). The explants were sterilized with chemical reagent from the result of experimental Part I and cultivated on MS and ½MS medium supplement with various concentration and combinations of auxins and cytokinins. So, this experiment have 32 treatments ; 5 replications per treatment. There were 20 bottles for each treatment with an explants in each (n = 100) . Incubation at 25 ^{o}C for 16 hours in light and 8 hours in dark. The results of explants were observed by regeneration rate.

The culture media used for this study were MS media and ½MS variously supplemented with different concentrations and combinations of auxins (2,4-D) and cytokinins (BAP) is shown in Table 3.2 and Table 3.3. Stock solution was prepared for the MS and ½MS medium, which can occur when the pH approaches 5.7 with 1N sodium hydroxide or 1N hydrochloric acid. Dissolving the calcium salt separately before adding it will also help to avoid precipitation (Gamborg and Phillips, 1995). Sucrose 30 g. and plant growth regulators stock solution were added and stirring continued until complete dissolution. Agar (0.7% w/v) was added and heated gently with continuous stirring until the solution cleared. The culture medium was sterilized by autoclaving at 121 ^{o}C, 15 lb/in^{2}, for 15 – 20 minutes (Chatsiriwej, 2004).

The culture media used for this study were MS and ½MS variously supplemented with different concentrations and combinations of four BAP concentrations (0, 10, 15 and 20 µM) and four 2,4-D concentrations (0, 1, 5 and 10 µM) for the regeneration of stem. The culture medium used consisted of MS and ½ MS medium containing 3% sucrose, 0.7% agar and medium were adjusted to pH 5.7 before autoclaving at 121 $^{\circ}$C for 20 min. The explants were incubated for 40 days at 25 $^{\circ}$C with photosynthetic photon flux densities (PPFD) of 0-40 µmol m^{-2} s^{-1} under 16 hours photoperiod. The morphological change of the leaf and nodal segments was recorded periodically after incubation in the same culture medium for 30 days. Five replications were used in each treatment with forty-five bottles in a treatment.

Table 3.2 Composition of MS medium used for shoot multiplication test and callus induction**.**

BA(µM) / **2,4-D(µM)**	**0**	**10**	**15**	**20**
0	C1	C2	C3	C4
1	C5	C6	C7	C8
5	C9	C10	C11	C12
10	C13	C14	C15	C16

Table 3.3 Composition of ½MS medium used for shoot multiplication test and callus induction.

BA(µM) / **2,4-D(µM)**	**0**	**10**	**15**	**20**
0	H1	H2	H3	H4
1	H5	H6	H7	H8
5	H9	H10	H11	H12
10	H13	H14	H15	H16

Table 3.4 Morphological characteristic and score level of callus growth

Score level	diameter of the callus (cm)
0	0-0.50
1	0.51-1.00
2	1.01-1.50
3	1.51-2.00
4	2.01-2.50
5	2.51-3.00
6	≥3.01

- Statistical analyses

A 4x4 factorial in CRD (Completely Randomized Design) was conducted to test. The results were compared with plant growth regulators-free media (MS and ½MS basal medium ; control treatments). Regeneration of stem explants was recorded for the increasing in diameter of the callus and scored, numbers of shoot per explants was recoded after 30 days. The mean value of treatment were compared using the Turkey's Studentized Range (HSD) Test at p less than 0.05.

3.3.3 Root Induction

For induction of adventitious roots, clumps of three to four microshoots (2–3 cm in height) were aseptically isolated from the proliferating shoot clusters and subcultured in MS basal medium, MS supplemented with indole-3-butyric acid (IBA) 20 µM, ¼MS and ½MS. Each treatment was replicated three times, there were five bottles for each treatment with three explants in each (n = 45). All the cultures were maintained by subculturing on the same medium at 3 weeks intervals under the same cultural conditions. Incubation at 25 ^{o}C for 16 hours in light and 8 hours in dark.

The composition of MS medium is shown in appendix A. Stock solution was prepared for the MS ½MS and ¼MS medium, the chemicals are dissolved in distilled or high demineralized water. In general, one compound was adding at a time to avoid precipitation. Dissolving the inorganic nitrogen sources of the major salts first will avoid precipitation between phosphate and calcium sources when added subsequently, which can occur when the pH approaches 6.0 with 1N sodium

hydroxide or 1N hydrochloric acid. Dissolving the calcium salt separately before adding it will also help to avoid precipitation (Gamborg and Phillips, 1995). Sucrose 30 g. were added and stirring continued until complete dissolution. Agar (0.7% w/v) was added and heated gently with continuous stirring until the solution cleared. The culture medium was sterilized by autoclaving at 121 ^{o}C, 15 lb/in^{2}, for 15 – 20 minutes (Chatsiriwej, 2004).

For induction of adventitious roots, clumps of three to four microshoots (2-3 mm in height) were aseptically isolated from the proliferating shoot clusters and subcultured in ¼MS, ½MS, MS and MS medium supplemented with indole-3-butyric acid (IBA) at 20 μM (Saswati *et al.*, 2006). All the cultures were maintained by subculturing on the same medium at 30 days intervals under the same culture conditions. Data on rooting of regenerated shoots in different types and concentrations of growth regulators were analyzed by least standard deviation.

- Statistical analyses

The number of root per shoot was recorded after 30 days of culture. The mean value of treatment were compared using the Turkey's Studentized Range (HSD) Test at p less than 0.05.

3.3.4 Gas chromatography-mass spectrometry (GC-MS) analysis of major metabolites between *in vitro* and fresh Plookao

A. Sample preparation

The comparison of major metabolites between *in vitro* and fresh Plookao was investigated by GC-MS. The first sub-culture, the shoot that growth completely in 30 days and fresh Plookao that growth in 30 days was air-dried with Hot Air Oven on 60°C for 24 hours or to dryness. The sample were mashed and extracted by reflux method using 3 grams dry weight per 100 ml methanol on water bath for 1 hour. The mixture was filtered while hot by Watman No. 1 and evaporated for crude extract. The crude extract was detected by GC-MS.

B. GC–MS analyses

GC–MS analyses were carried out with electron impact ionization mode by using 1 µL. of the sample. A Zebron ZB-5 (5% phenyl-95% dimethyl-polysiloxane) column (30m×0.25mm×0.25 m) was used with an oven program of 80–240 ◦C at 3 ◦C/min for the first 20 min and 1 ◦C/min for the rest time. The final temperature was held for 30 min. The injector temperature was 240 ◦C. The flow-rate of the carrier gas (He, 99.9995%) was 25 ml/min. A split injection with a ratio of 1:20 was used.