CHAPT40ER 4
RESULTS AND DISCUSSION

4.1 Explants sterilization

The result of explants sterilization of *Houttuynia cordata* Thunb. was compared between leaf, stem and rhizome sterilized the NaOCl at 2%, 4% and 6% (v/v) and $HgCl_2$ at 0.1%, 0.3%, 0.5% (w/v), at 5 and 10 minutes. The explants were detected for contamination and percentage of regeneration.

A. Leaf sterilization

The result of sterilization showed that $HgCl_2$ at 0.3% and 0.5% for 5 and 10 minute gave no contamination on the leaf explants as shown in Table 4.1. Low contamination on the explants was obtained from 0.1% $HgCl_2$ for 10 minute and 6% 10 minute NaOCl after 10 days incubation. Microorganism contamination found in the leaf was both fungi and bacterial contamination. A physical characteristic of leaf sterilized with NaOCl was shown in Figure 4.1 and that of leaf sterilization with $HgCl_2$ was shown in Figure 4.2. It was shown that low concentration of disinfectant present high contamination, but high concentration used injuried the explants. Leaf explants is the popular part of the plant for tissue culture. Many report have successfully for cultured of leaf such as Maria and Aekaterini (2008) study the effect of position and orientation of leaflet explants with respect to plant growth regulators on micropropagation of *Zamioculcas zamiifolia* Engl. (ZZ), Seetharam *et al*. (2006) report in *in vitro* shoot regeneration from leaf and nodal explants of *Vernomia cinerea* (L.) Less. And the report of Gajakosh *et al*. (2010) to study the organogenesis from shoot tip and leaf explants of *Morinda Citrifolia* L. an important medicinal tree. They are one of the plant leaves very successful *in vitro*, but a depend on the characteristic of the leaves, such as a thickness, hairy, surface and toleration of disinfectant (Gamberg, 2002).

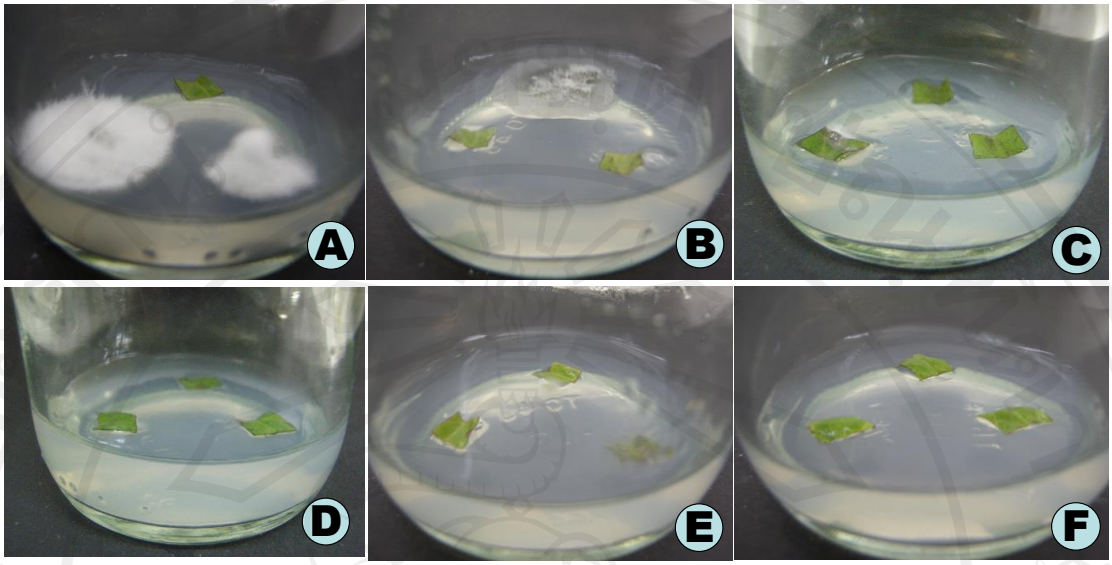

Figure 4.1 Leaf sterilized with NaOCl (A) 2% for 5 min. (B) 4% for 5 min. (C) 6% for 5 min. (D) 2% for 10 min. (E) 4% for 10 min. and (F) 6% for 10 min.

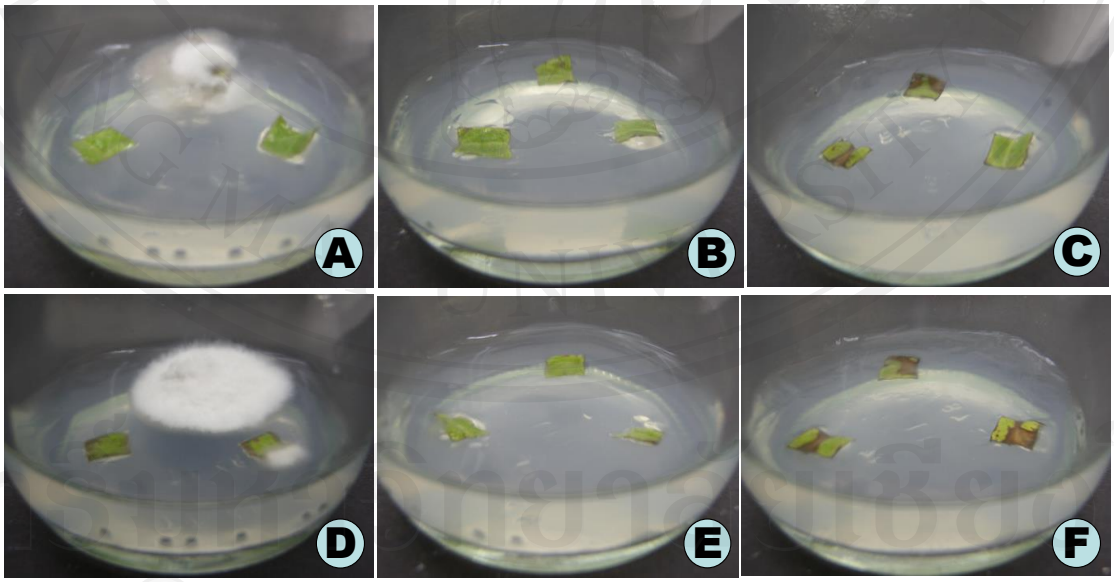

Figure 4.2 Leaf sterilized with $HgCl_2$ (A) 0.1% for 5 min. (B) 0.3% for 5 min. (C) 0.5% for 5 min.(D) 0.1% for 10 min. (E) 0.3% for 10 min. and (F) 0.5% for 10 min.

B. Stem sterilization

The result of stem sterilization showed that at 0.5% $HgCl_2$ for 10 minute, no contamination was present using 6% NaOCl for 10 minute, 0.5% $HgCl_2$ for 5 minute and 0.3% $HgCl_2$ for 10 minute gave low contamination. The result after sterilization and 10 day incubation, was shown in Table 4.1. And physical properties of stem sterilized with NaOCl was shown in Figure 4.3 and Figure 4.4, respectively. The data shown that stem sterilization with NaOCl got highly contamination than sterilized with $HgCl_2$ and the stem explants was more tolerance to disinstant then the leaf explants.

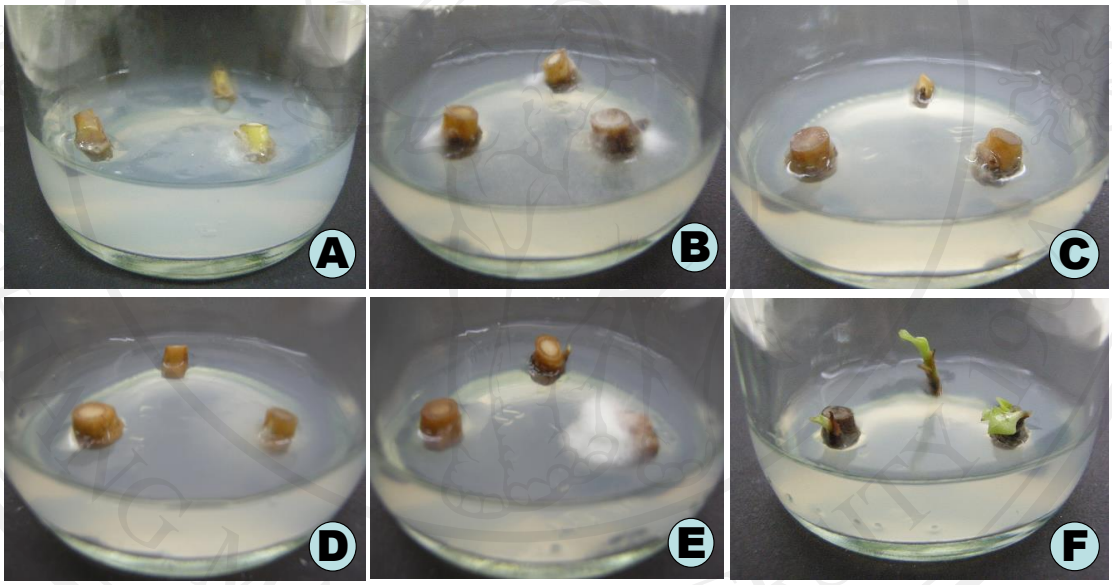

Figure 4.3 Stem sterilized with NaOCl (A) 2% for 5 min. (B) 4% for 5 min. (C) 6% for 5 min. (D) 2% for 10 min. (E) 4% for 10 min. and (F) 6% for 10 min.

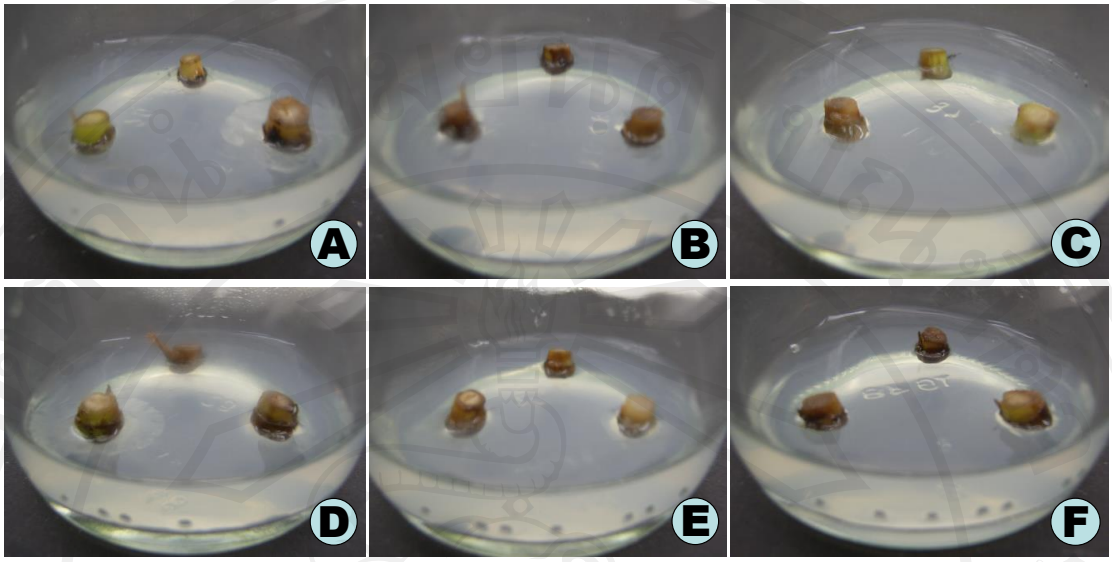

Figure 4.4 Stem sterilized with $HgCl_2$ (A) 0.1% for 5 min. (B) 0.3% for 5 min. (C) 0.5% for 5 min.(D) 0.1% for 10 min. (E) 0.3% for 10 min. and (F) 0.5% for 10 min.

C. Rhizome sterilization

The result of rhizome sterilization showed that $HgCl_2$ at 0.5% for 10 minute have no contamination. Sterilization with $HgCl_2$ at 0.3% for 10 minute, 0.5% for 5 minute 0.1 for 10 minute and NaOCl at 6% for 10 minute was low contamination after 10 day incubation. as shown in Table 4.1. Picture of physical of rhizome sterilized with NaOCl and $HgCl_2$ was shown in Figure 4.5 and in Figure 4.6, respectively. It was found that rhizome sterilized with NaOCl can be regenerated when incubated for 15 days.

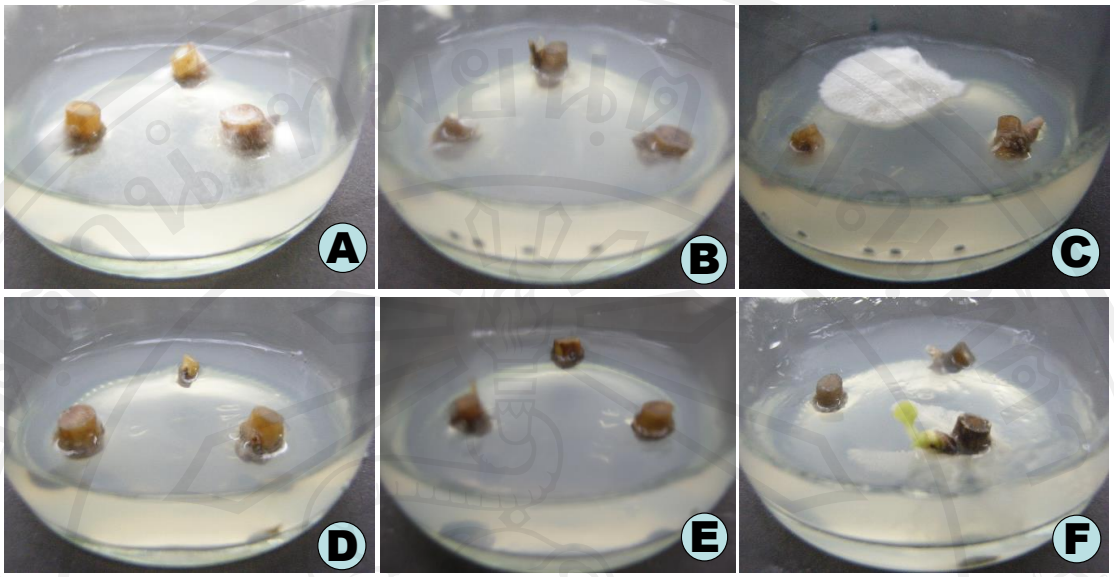

Figure 4.5 Rhizome sterilized with NaOCl (A) 2% for 5 min.(B)4% for 5 min.(C)6% for 5 min. (D) 2% for 10 min. (E) 4% for 10 min. and (F) 6% for 10 min.

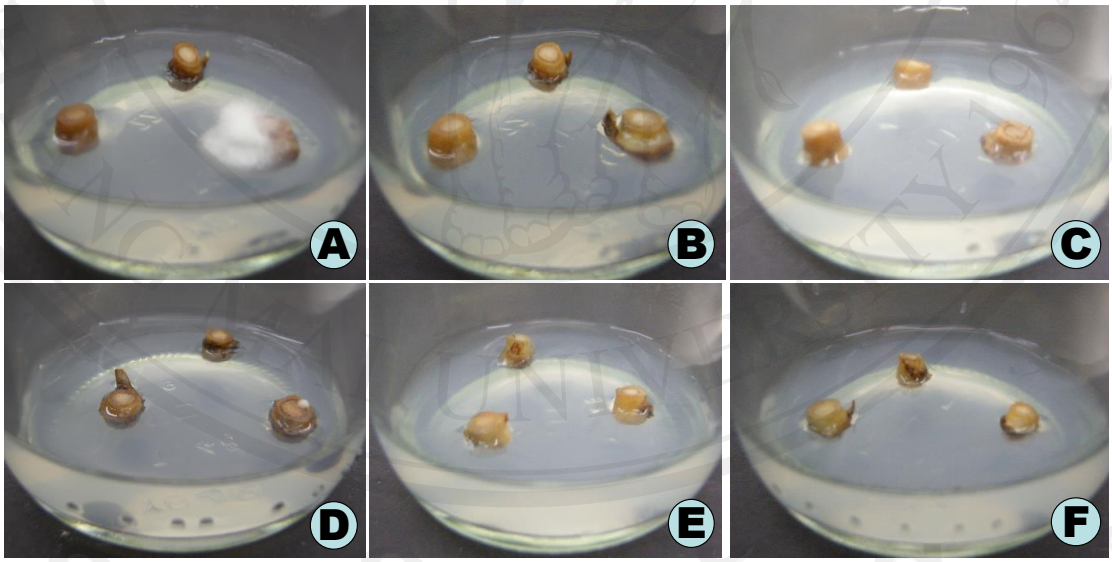

Figure 4.6 Rhizome sterilized with $HgCl_2$ (A) 0.1% for 5 min. (B) 0.3% for 5 min. (C) 0.5% for 5 min.(D) 0.1% for 10 min. (E) 0.3% for 10 min. and (F) 0.5% for 10 min.

Table 4.1 Comparison of contamination in leaf, stem and rhizome explants.

Explants	Sterilizing Agent	Concentration (%)	Sterilization Time	
			5 min	10 min
Leaf	NaOCl	2	31.06±0.08^{c}	28.87±0.08^{c}
		4	26.63±0.14^{bc}	19.87±0.12^{abc}
		6	17.73±0.08^{abc}	6.63±0.07^{ab}
	$HgCl_2$	0.1	11.07±0.03^{abc}	6.63±0.07^{ab}
		0.3	0.00±0.00^{a}	0.00±0.00^{a}
		0.5	0.00±0.00^{a}	0.00±0.00^{a}
Stem	NaOCl	2	51.10±0.10^{bc}	66.63±0.07^{c}
		4	62.20±0.16^{c}	22.20±0.10^{ab}
		6	48.87±0.10B^{c}	6.63±0.07^{a}
	$HgCl_2$	0.1	28.87±0.08^{ab}	17.73±0.14^{a}
		0.3	22.20±0.10^{ab}	8.87±0.08^{a}
		0.5	6.67±0.12^{a}	0.00±0.00^{a}
Rhizome	Clorox	2	57.73±0.10^{e}	55.53±0.14^{e}
		4	46.63±0.18^{de}	33.30±0.07^{bcde}
		6	35.50±0.10^{bcde}	24.43±0.08^{abcd}
	HgCl2	0.1	39.97±0.07^{cde}	24.43±0.14^{abcd}
		0.3	31.07±0.03^{bcde}	13.30±0.07^{abc}
		0.5	8.87±0.08^{ab}	0.00±0.00^{a}

* Values represent mean ± standard error of 5 replicates treatment. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

From the data on Figure 4.3 and 4.4, it was found that stem explants was more tolerance to disinfectant then the other types of explants and lower contamination was found in the $HgCl_2$ treatment compared to NaOCl. However, only stem and rhizome can regenerated in this experiment after 10 days incubation with the highest number at 82.96 % and 73.33 % from stem and rhizome respectively (Table 4.4) . Moreover, the explants sterilization with $HgCl_2$ treatment have been reported in several plant species such as *Sesamum indicum* L. (Saravanan and Nadarajan, 2005), Cucumis sativus L. (Jin-Feng *et al.*, 2009), Rubus L. (Sandhya and Mahalaxmi, 2009).

In our environment, different seasons has a profound effect on plant physiology. The result found that stem and rhizome explants showed the lowest contamination when explants collected during summer and winter season (Data not shown).

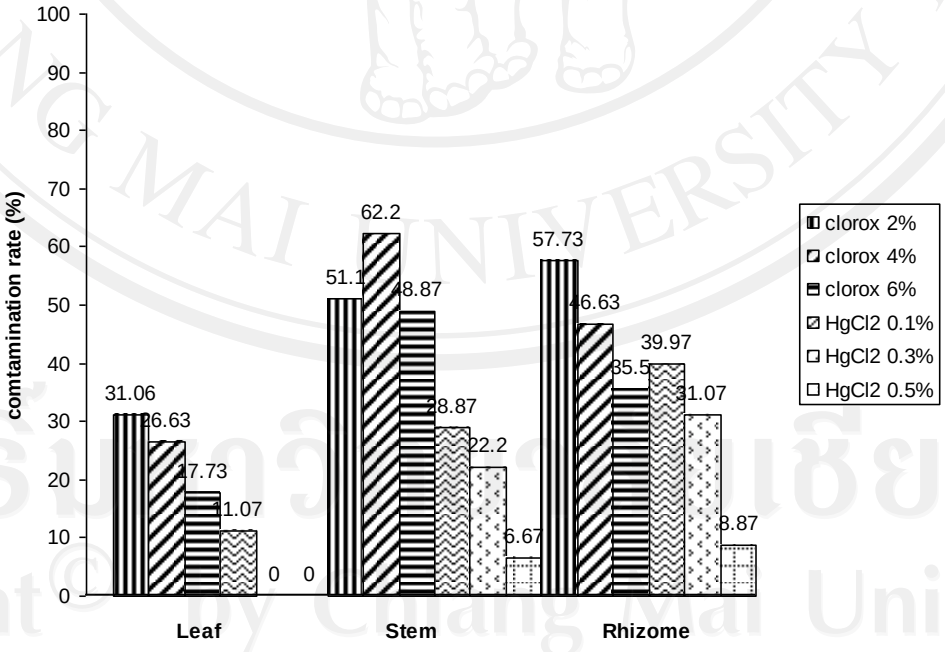

Figure 4.7 Comparison of number of contamination between explants after sterilization for 5 minutes.

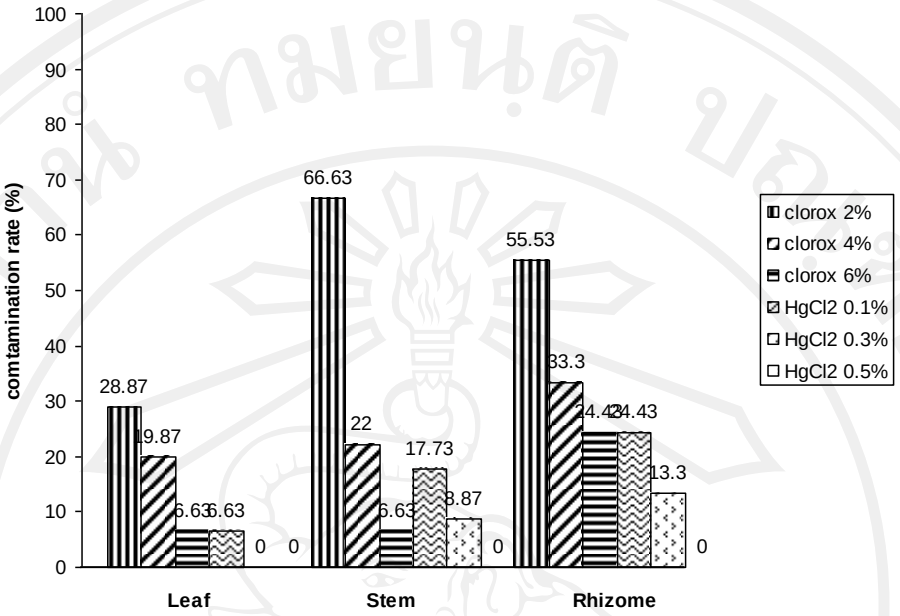

Figure 4.8 Comparison of number of contamination between explants after sterilization for 10 minutes.

D. The regeneration of Explants

The percentage of regeneration of explants in MS media after incubation for 15 days was shown in Table 4.2. It was found that regeneration of explants sterilized with NaOCl were 60.98% on stem and 41.38% on rhizome and the highest number was obtained from $HgCl_2$ sterilization at 82.96 % and 73.33 % from stem and rhizome respectively. The new shoot from stem explants was green color shown in Figure 4.9. The result for explants regeneration on MS basal medium without plant growth regulators was shown in many reported. For example,

Mahendran and Narmatha (2009) established *Datyrium nepalense* D.Don culture by cutting immature seeds from unripe fruits and were germinated on MS medium. The germination of the seeds and development of protocorm was highest in MS 86.7%.

Gaba *et al.* (2009) found that the regeneration of *Cucurbita pepo* L. from cotyledon explants cultivars is generally efficient on Murashige and Skoog.

Figure 4.9 Show the regeneration from stem explants on MS media.

Table 4.2 The percentage of regeneration rate of explants

Treatment		Explants		
Time	**Concentration**	**Leaf**	**Stem**	**Rhizome**
NaOCl (5 minute)	2%	-	8.87±0.23^{cd}*	6.67±0.06^{d}
	4%	-	6.25±0.06^{d}	4.35±0.06^{d}
	6%	-	4.55±0.06^{d}	10.71±1.21^{cd}
NaOCl (10 minute)	2%	-	21.22±0.66^{c}	10.53±1.21^{cd}
	4%	-	60.98±2.00^{b}	41.38±1.99^{bc}
	6%	-	20.59±0.58^{c}	21.21±0.58^{c}
HgCl2 (5 minute)	0.1%	-	8.87±1.01^{cd}	6.67±0.66^{d}
	0.3%	-	6.25±0.25^{d}	4.35±0.51^{d}
	0.5%	-	4.55±0.51^{d}	10.71±1.27^{cd}
HgCl2 (10 minute)	0.1%	-	82.96±1.99^{a}	73.33±1.01^{a}
	0.3%	-	6.25±0.25^{d}	4.35±0.58^{d}
	0.5%	-	-	-

* Values represent mean ± standard error of 5 replicates treatment. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

4.2 Shoot multiplication and callus induction

- Stem explants were collected in this experiment. Initiation of aseptic culture. Stem explants were surface sterilized with 0.1% $HgCl_2$ solution for 10 min, rinsed thoroughly in sterile distilled water, and cut slide into 1 cm long section.

A. Shoot multiplication

Evaluation of the explants preparation conducted by slide cut (45 degree) and straight cut of the stem at 0.5 and 1.0 cm long. The result showed that slide cut at 1.0 cm long gave the best result with the longest shoot length at 2.41 cm. after 20 days cultivation (data not shown).

The effect of media and plant growth regulators on regeneration of the stem explants was shown in Table 4.3 and 4.4. The number of shoot per explants increased when concentration of BAP increased within 30 days of incubation. The highest number of shoot per explants was 19.40 shoot in the MS medium supplemented with 10 µM BAP alone. The shoot in MS medium present a normal and healthy form of stem and leaf compared to the one in ½MS (Figure 4.10 and 4.11).

The frequency of axillary shoot proliferation and the number of shoots per explants increased with increasing concentration of cytokinin up to an optimum level (Table 4.3 and 4.4). BAP at a concentration of 10 µM induced multiple shoot buds in virtually explants within 30 days. The buds appeared as small green protuberances in the nodal explants that elongated into slender shoot processing diminutive leaves (Figure 4.10 and 4.11). The highest number of shoots per explants was recorded to be 19.40±3.31, on which formed in the MS medium supplemented with 10 µM BAP alone. However the frequency of shoot proliferation in the ½MS media supplement with BAP alone was relatively low and there were fewer shoots per explants compare with the MS media supplement with BAP alone. BAP induced axillary shoot proliferation have been reported in several plant species such as *Houttuynia cordata* Thunb. (Rabindra, 2006), *Wrightia tinctoria* R.Br. (Purohit and Kunda, 1994), *Arachis hypoaea* L. (Saxena *et al*., 1992).

Table 4.3 Effect of ½MS medium supplemented with either 2,4-D and BAP or alone on the number of shoot multiplication per explants from nodal explants of Plookao after 30 days of culture

2,4-D \ BA	**0**	**10**	**15**	**20**
0	H1= 1.00 ± 0.00[a]*	H2= 10.00±2.84 [b]	H3= 7.21±2.53 [b]	H4= 3.99±2.03[ab]
1	H5= 0.00±0.00 [a]	H6= 0.00±0.00 [a]	H7= 0.00±0.00 [a]	H8= 0.00±0.00 [a]
5	H9= 0.00±0.00 [a]	H10= 0.00±0.00 [a]	H11= 0.00±0.00 [a]	H12= 0.00±0.00 [a]
10	H13= 0.00±0.00 [a]	H14= 0.00±0.00 [a]	H15= 0.00±0.00 [a]	H16= 0.00±0.00 [a]

* Values represent mean ± standard error of 5 replicates treatment. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

Table 4.4 Effect of MS medium supplemented with either 2,4-D and BAP or alone on the number of shoot multiplication per explants from nodal explants of Plookao after 30 days of culture

2,4-D \ BA	**0**	**10**	**15**	**20**
0	C1= 1.00±0.00 [a]*	C2= 19.40±3.31[c]	C3= 7.80±2.61[ab]	C4= 5.80±1.33[ab]
1	C5= 0.00±0.00 [a]	C6= 0.00±0.00 [a]	C7= 0.00±0.00 [a]	C8= 0.00±0.00 [a]
5	C9= 0.00±0.00 [a]	C10= 0.00±0.00 [a]	C11= 0.00±0.00 [a]	C12= 0.00±0.00 [a]
10	C13= 0.00±0.00 [a]	C14= 0.00±0.00 [a]	C15= 0.00±0.00 [a]	C16= 0.00±0.00 [a]

* Values represent mean ± standard error of 5 replicates treatment. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

Figure 4.10 Shoot formation from stem explants incubated on ½ MS medium supplement with 10 µM BA alone for 30 days

Figure 4.11 Shoot formation from stem explants incubated on MS medium supplement with 10 µM BA alone for 30 days

B. Callus induction

Stem with a nodal segment was cultured on ½MS and MS media supplement with various concentration of BAP combined with 2,4-D with in 30 days of inoculation in media supplemented with varied plant growth regulators, callus was initiated from cut edge of the explants, on which 100% of the explants formed callus. Callus were solf, Green-Yellowish or green gray in color with adventitious shoot buds differentiated from the surface see Figure 4.12, easy to break into small pieces when touched. They were fromed on both ½MS and MS medium supplement with 10 µM BAP combined with 1µM 2,4-D. However, the growth of calluses was rather slow while the ½MS and MS medium supplemented with lower and higher 10 µM BAP combined with 1µM 2,4-D (Table 4.5 and 4.6).

Table 4.5 Effect of ½MS medium supplemented with either 2,4-D and BAP or alone on the score of callus induction from nodal explants of Plookao after 30 days of culture

BA / **2,4-D**	**0**	**10**	**15**	**20**
0	H1= 0.00 ± 0.00[a]*	H2= 0.00 ± 0.00[a]	H3= 0.00 ± 0.00[a]	H4= 0.00 ± 0.00[a]
1	H5= 1.67 ± 0.11[b]	H6= 4.07 ± 0.13[e]	H7= 3.27 ± 0.13[e]	H8= 3.33 ± 0.13[e]
5	H9= 3.40 ± 0.13[e]	H10= 2.33 ± 0.13[b]	H11= 2.60 ± 0.13[b]	H12= 1.80 ± 0.11[b]
10	H13= 2.67 ± 0.13[b]	H14= 1.67 ± 0.13[b]	H15= 1.00 ± 0.00[a]	H16= 2.67 ± 0.13[b]

* Values represent mean ± standard error of 5 replicates treatment. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

Table 4.6 Effect of MS medium supplemented with either 2,4-D and BAP or alone on the score of callus induction from nodal explants of Plookao after 30 days of culture

2,4-D \ BA	**0**	**10**	**15**	**20**
0	C1= 0.00±0.00 [a]*	C2= 0.00±0.00 [a]	C3= 0.00±0.00 [a]	C4= 0.00±0.00 [a]
1	C5= 1.81 ± 0.10[e]	C6= 5.71 ± 0.12[e]	C7= 3.47 ± 0.13[d]	C8= 3.43 ± 0.13[d]
5	C9= 3.40 ± 0.13[d]	C10= 3.40 ± 0.13[d]	C11= 1.87 ± 0.09[b]	C12= 2.87 ± 0.13[b]
10	C13= 1.80 ± 0.11[b]	C14= 1.87 ± 0.09[b]	C15= 1.80 ± 0.11[b]	C16= 1.00 ± 0.00[a]

* Values represent mean ± standard error of 5 replicates treatment. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

The highest score of callus (5.71 ± 0.12) were record at MS medium supplement with 10 µM BAP combined with 1µM 2,4-D (Table 4.6). It is the same to the earlier reports where the higher concentration of BAP combination with auxin enhanced the callus formation (Vengadesan, 2000) and the report of Thanpicha, 2007 to cultured *Mucuna macrocarpa* Wall. The result shown the highest growth and size increasing were observed in the callus cultured on MS medium containing 2.0 mg/l 2,4-D and 1.0 mg/l BAP.

Figure 4.12 Green-Yellowish friable callus in the MS medium with adventitious shoot buds differentiated from the surface

4.3 Root induction

In vitro raised roots with microshoots (2-3 cm.) were excised from the proliferating shoot cluster and placed on ¼MS, ½MS, MS and MS medium supplemented with indole-3-butyric acid (IBA) at 20 μM. Among the treatments, IBA alone produced better root induction than ¼ MS, ½ MS, MS medium. Optimal root induction was observed on 20 µM IBA alone (Table 4.7). Such an efficiency of IBA on rooting was also reported in other species (Sugiura *et al*., 1986; Winnaar, 1988; Rani *et al*., 2003; Saha *et al*., 2003). And Saswati *et al*. (2006) report the effect of different concentration and combination of plant growth regulators on root induction found that optimal root induction was observed on IBA alone. The study of Methinee *et al*. (2009) showed that shoots which cultured on 10-20 µM IBA gave the highest root number of 3.4-4.7 roots and root induction was 100 %. The root growth from nodal region of the microshoots and hairy root formation from the nodal region is a characteristic feature of the stoloniferous rhizome of Plookao (Figure 4.13).

Table 4.7 Effect of media concentration and combinations of plant growth regulators on root induction and growth after 20 days of culture

condition	No. of roots per shoot	Root length (mm)
MS	3.43 ± 2.19[b]*	6.56 ± 2.05[a]
½ MS	4.00 ± 1.20[b]	5.20 ± 1.43[ab]
¼ MS	1.43 ± 1.79[bc]	2.06 ± 1.66[c]
MS+20 µM IBA	7.21 ± 1.61[a]	9.00 ± 1.75[a]

*Each value in the column represents mean ±SD of five replications. Mean values followed by the same letter are not significantly different by the Turkey's test at 0.05 propability level.

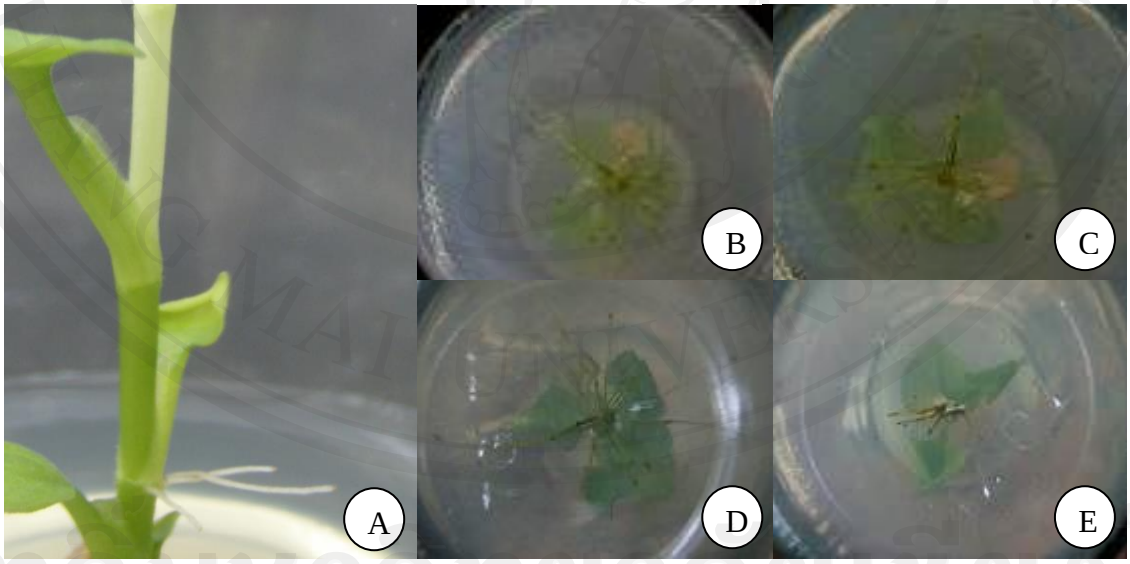

Figure 4.13 Hairy roots formation from the node of differentiated shoots A) MS B) ½MS C)-D) MS + IBA 20 μM and E) ¼MS

4.4 Gas chromatography-mass spectrometry (GC-MS) Analysis between fresh and *in vitro* Plookao

The analysis between fresh and *in vitro* culture of Plookao crude extract and fresh and *in vitro* Plookao distillation by using GC-MS was found that the chromatogram of fresh Plookao crude extract (Fig 4.14-4.15) show the peak of chemical that is Caryophyllene on time 15.31 and Phytol on time 24.55. *In vitro* Plookao crude extract was found that Bornyl acetate on time 13.04, Caryophyllene on time 15.31, Oxirane on time 21.19 and 1,2-Benzenedicarboxylic acid, Di-isooctyl ester on time 29.11. The result indicated that plant of the same age but from different sources present different types of major metabolite and further investigation will be useful for the pharmaceutical application.

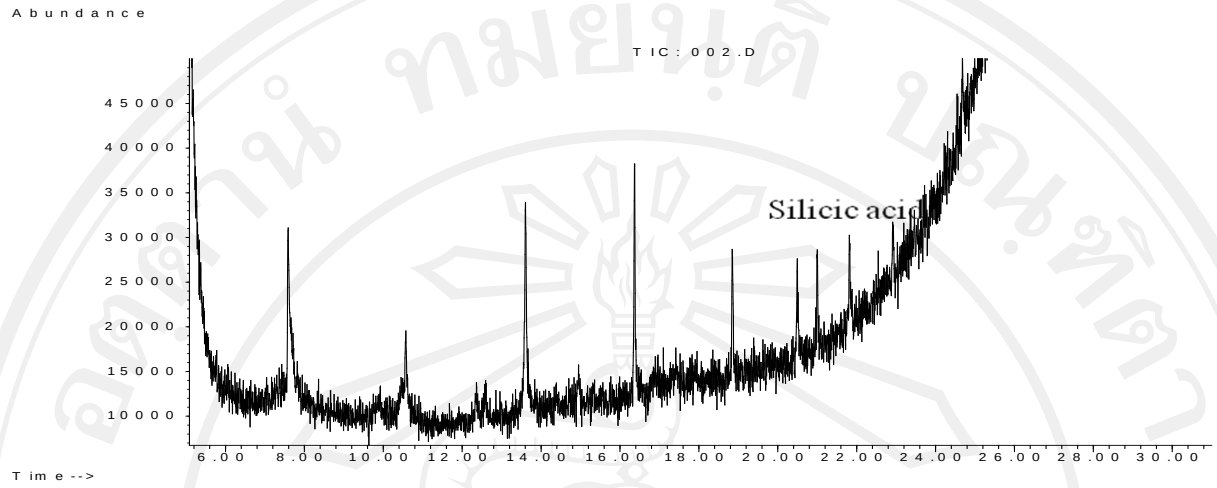

Figure 4.14 Chromatograms of ditillation of Fresh Plookao by GC-MS under the extraction condition of 70 ◦C and 60 min by Reflux method

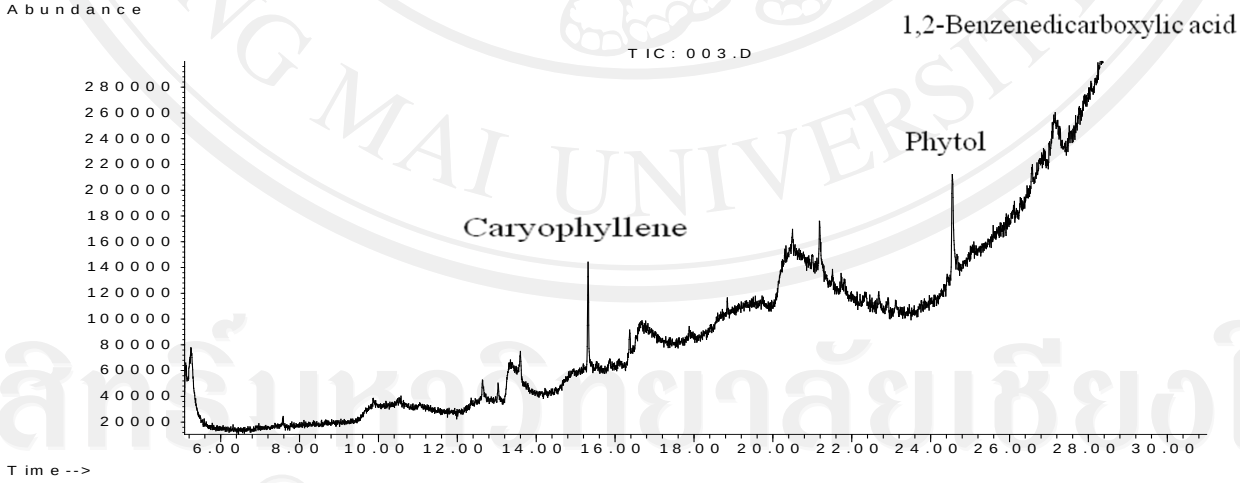

Figure 4.15 Chromatograms of crude extract of Fresh Plookao by GC-MS under the extraction condition of 70 ◦C and 60 min by Reflux method

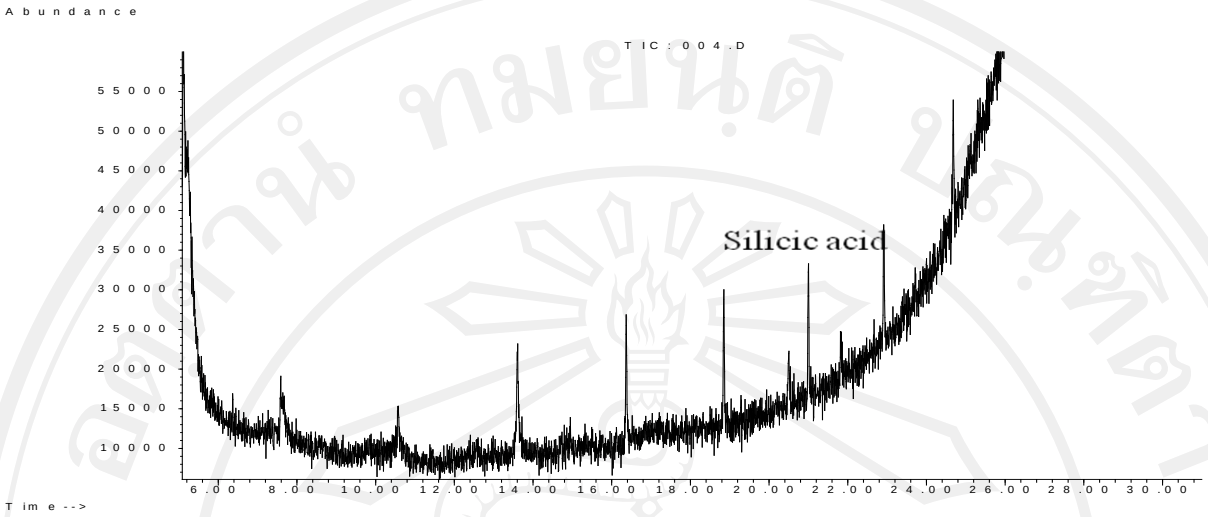

Figure 4.16 Chromatograms of distillation of *In vitro* Plookao by GC-MS under the extraction condition of 70 ◦C and 60 min by Reflux method

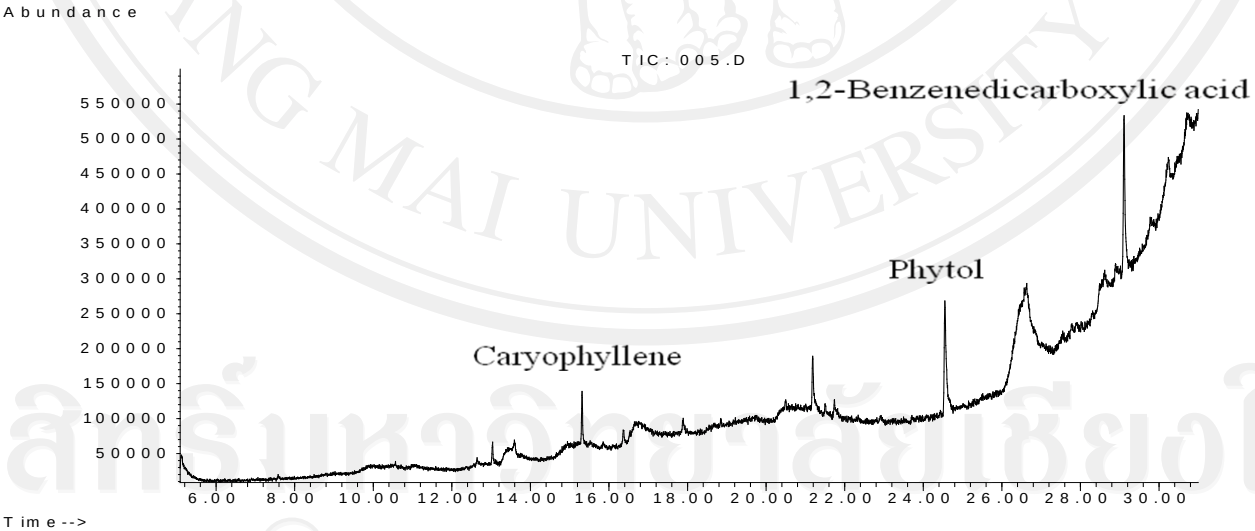

Figure 4.17 Chromatograms of crude extract of *In vitro* Plookao by GC-MS under the extraction condition of 70 ◦C and 60 min by Reflux method

Table 4.8 Show major ingredient from fresh and *in vitro* Plookao by GC-MS

Condition	Active ingredient	Time (min)	Quality (%)	Correlation area
Fresh Plookao distillation	Glaucine	18.85	27	302784
	Indole	20.49	38	301482
	Silicic acid	21.83	37	304143
Fresh Plookao crude	Phenol	12.64	25	475816
	1,3-Benzenediol	13.04	22	645687
	Caryophyllene	15.32	97	1197119
	Pyrazine	16.37	22	370443
	7-Oxabicyclo[4.1.0]heptane	21.19	30	301436
	Phytol	24.55	58	2587525
	1,2-Benenedicarboxylic acid	29.11	80	4873274
In vitro Plookao distillation	Benzothiophene-3-carboxylic	20.49	80	686457
	Silicic acid	21.82	32	313464
	Vitamin E	30.10	94	1215212
In vitro Plookao crude	Dichloropropylphosphine	12.64	27	473425
	Acetic acid	13.04	95	492213
	2-propanamine	13.60	25	645687
	Caryophyllene	15.32	95	1117222
	Oxirane	21.19	60	1314172
	Stibine	21.50	46	301436
	Phytol	24.55	96	4279097
	Stigmasterol	26.58	90	684219
	1,2-Benendicarboxylic acid	29.11	47	5676247

The data from Table 4.8 show the active ingredient from fresh and *in vitro* Plookao. There were 3 major components found in fresh Plookao ditillation including Glaucine, Indole and Silicic acid and from fresh Plookao crude extract 7 major component such as Phenol, 1,3-Benzenediol, Caryophyllene, Pyrazine, 7-Oxabicyclo[4.1.0]heptanes, Phytol and 1,2-Benenedicarboxylic acid. *In vitro* Plookao distillation was composed of 3 major components of Benzothiophene-3-carboxylic, Silicic acid and Vitamin E. There were 9 major components found in *in vitro* Plookao crude extract including Dichloropropylphosphine, Acetic acid, 2-propanamine, Caryophyllene, Oxirane, Stibine, Phytol, Stigmasterol and 1,2-Benendicarboxylic acid. The chromatogram of fresh Plookao crude extract show three peak of the same chemical found in *in vitro* Plookao crude extract including Caryophyllene, Phytol and 1,2-Benendicarboxylic acid and the chromatogram of fresh Plookao ditillation show one peak of the same chemical found in *in vitro* Plookao ditillation such as Silicic acid. The correlation area (Indicates the amount of the substance but not calculated as the quantity, since no standard for comparison) shown almost of the same major components found in *in vitro* Plookao more than fresh Plookao except Caryophyllene.