CHAPTER IV

RESULTS

1. Development of TLC-Densitometry for the Determination of Plaunotol in *C. sublyratus* Leaves

In order to screen for *C. sublyratus* plants with high plaunotol content, it is necessary to have an assay method which is simple, rapid and accurate. Thin-layer chromatography (TLC) is a method for separation of components in a plant crude extract. This technique also allows various components separated on a TLC plate be quantitated by using an instrument called densitometer which is capable of scanning the separated spots on the TLC plate to produce a chromatogram. Recently, this TLC-densitometric method has been applied to the quantitative analysis of a wide variety of chemicals. For example, a simultaneous determination of individual curcuminoids in turmeric by TLC-densitometric method (Supinya Tewtrakul *et al.*, 1992).

The determination of plaunotol in *C. sublyratus* leaves has been reported to be carried out by using gas chromatography (Morimoto and Murai, 1989; Ogiso *et al.*, 1981; Sununta Cajesanun, 1991). This method is, however, complicated and time-consuming. We, therefore, in this study developed a new method of TLC-densitometry for determination of plaunotol in *C. sublyratus* leaves.

1.1 TLC Separation of Plaunotol in *C. sublyratus* Crude Extracts

A preliminary study showed that the solvent system of chloroform and n-propanol, 24:1, resulted in a good separation on a TLC (Silica gel) plate of plaunotol from other components in the ethanolic extract of *C. sublyratus* leaves. However, scanning of the TLC plate using the wavelength of 220 nm showed that the peak of plaunotol in the TLC densitometric chromatogram was not completely separated from the other components (Fig. 9A). Therefore, an improvement to obtain a better TLC separation of plaunotol was carried out. By varying various ratio of chloroform : n-propanol and using the technique of repeating TLC development, it was found that the complete separation of plaunotol from the other components could be achieved by triple development of two solvent systems run consecutively in one dimension of the TLC plate. The first development used the solvent system of chloroform : n-propanol (24:1), the second and third developments used chloroform : n-propanol (24:0.5). The results of the consecutive TLC densitometric chromatograms are shown in Fig. 9. Under these conditions, the final Rf value of plaunotol was found to be 0.28.

1.2 Identification of Plaunotol on TLC Plate

To confirm that the peak of plaunotol in the TLC densitometric chromatogram was absolutely contributed by pure plaunotol, the spot on the TLC plate corresponded to the Rf value of starndard plaunotol was scanned to produce a UV-absorption spectrum. The obtained UV-absorption spectrum was then compared with that of authentic plaunotol. As shown in Fig. 10, it was found that plaunotol in the ethanolic extract and standard plaunotol had identical absorption spectrum with their λmax at 220 nm. Therefore, it was clear that the peak with the Rf value of 0.28 on the TLC

plate was contributed by only pure plaunotol present in the ethanolic extract of *C. sublyratus* leaves. Furthermore, based on the UV-absorption spectrum, the wavelength of 220 nm appeared to be suitable for performing quantitative analysis of plaunotol in *C. sublyratus* leaves by TLC-densitometric method.

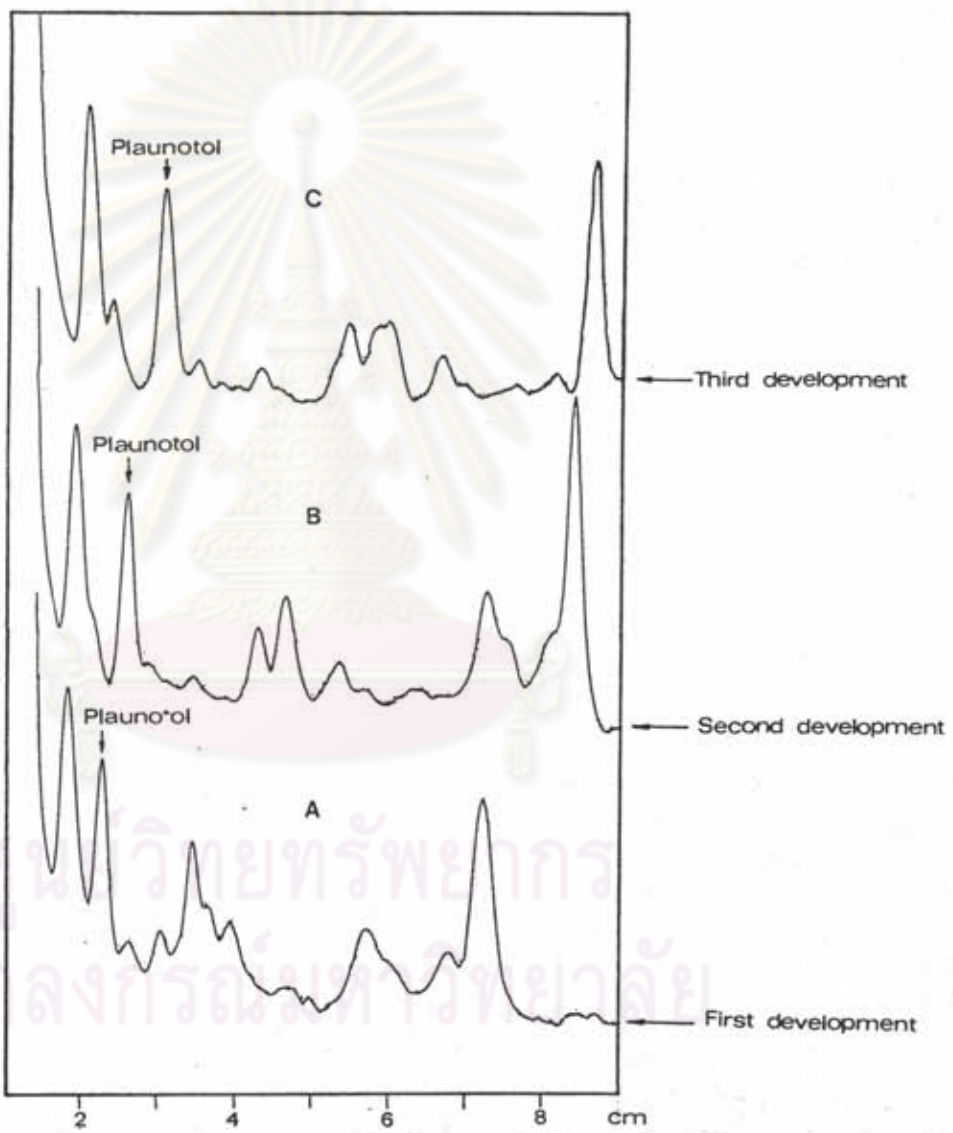

Figure 9 TLC-chromatogram of plaunotol in ethanolic extract of *C. sublyratus* leaves after ascending triple development in solvents.

A) first development - chloroform : n-propanol (24:1)

B) second development - chloroform : n-propanol (24:0.5)

C) third development - chloroform : n-propanol (24:0.5)

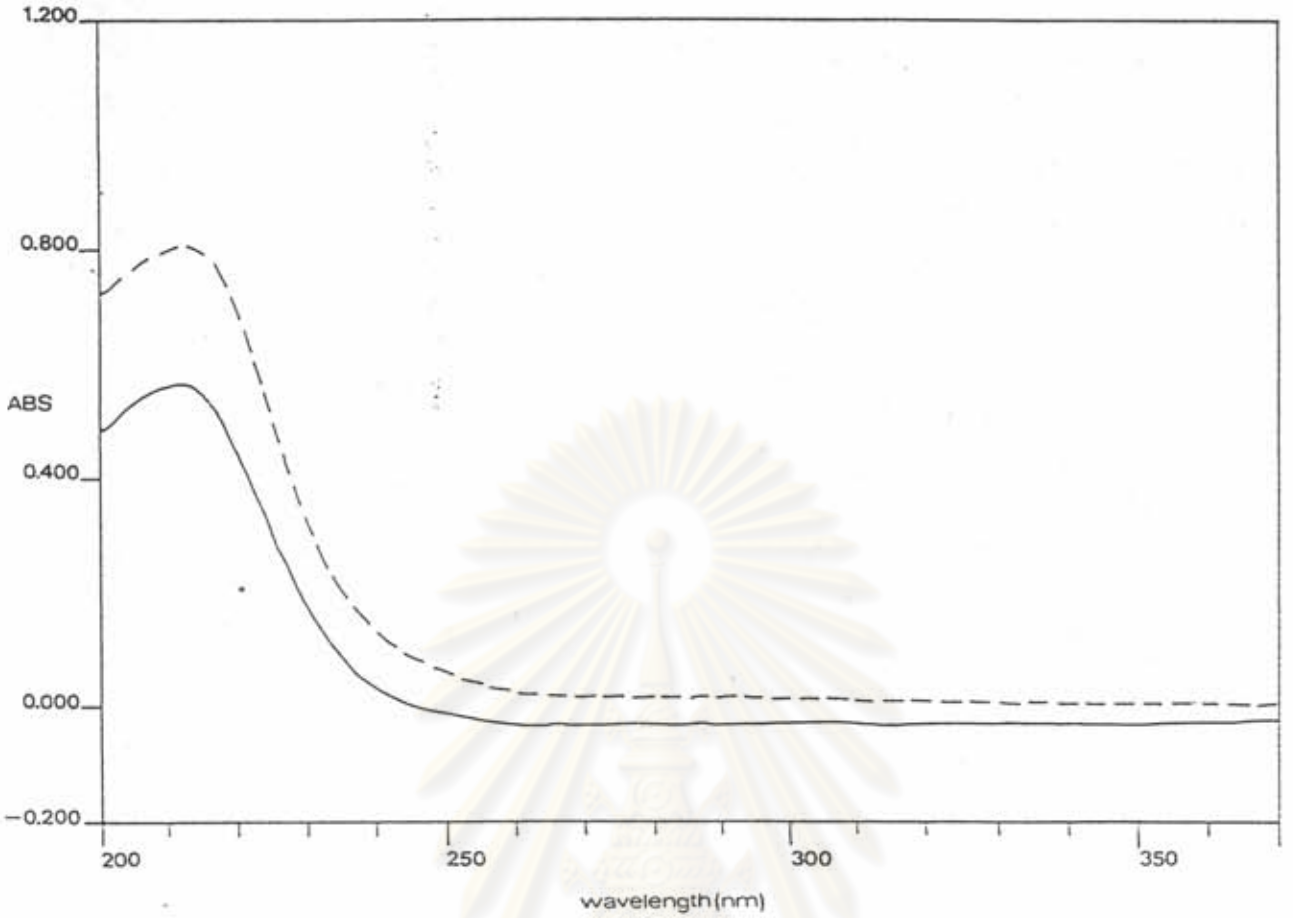

Figure 10 UV-absorption spectra of authentic plaunotol (----) and the compound on TLC plate with similar Rf-value to plaunotol which was separated from the ethanolic extract of *C. sublyratus* leaves (_____).

1.3 Standard Calibration Curve

The complete separation of plaunotol from other leaf constituents by the triple development of TLC plate allowed the compound be quantitated by the method of densitometry using the calibration curve of authentic plaunotol for calculation. The calibration curve of standard plaunotol which was obtained by plotting the peak areas against plaunotol concentrations is shown in Fig. 11. This curve showed linearity of the relationship between 0.125 to 1.0 μg for a 5 μl application volume of each plaunotol concentration which was equivalent to 0.025 to 0.2 mg plaunotol per ml. The regression analysis and the correlation coefficient was found to be 0.998.

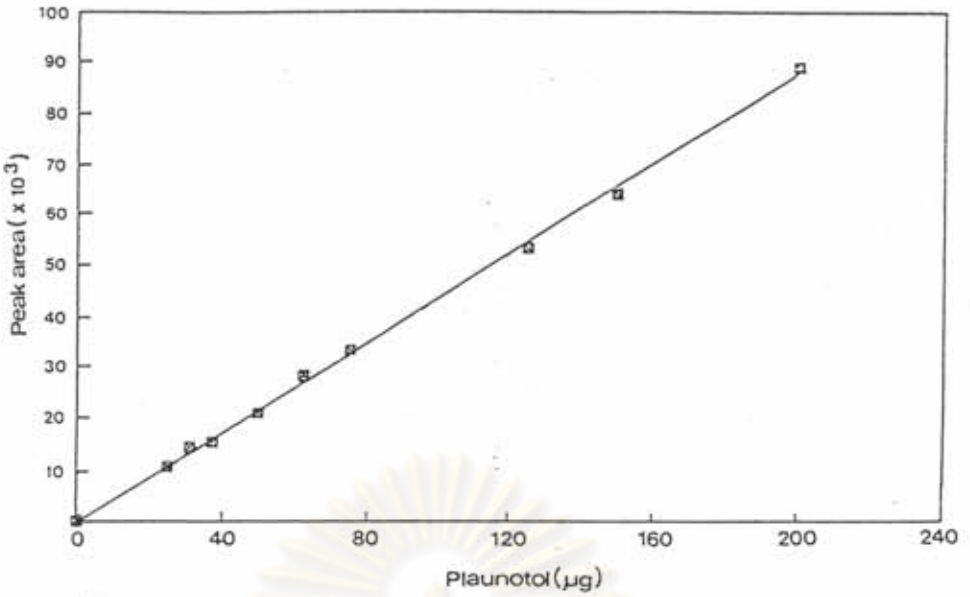

Figure 11 Calibration curve of plaunotol by TLC-densitometric method.

1.4 Accuracy and Precision

In order to evaluate its accuracy and precision, the developed TLC-densitometry was compared with the method of gas chromatography (GC) which was described by Sununta Cajesanun (1991) and in the Materials and Methods (Section 2.4). The separation of leaf plaunotol by GC is shown in Fig. 12. Its retention time appeared in the chromatogram was found to be approximate 3.4. The calibration curve of plaunotol obtained by the GC method (Fig. 13) showed linearity of the relationship from 0.05 to 0.8 mg/ml and the correlation coefficient was found to be 0.998. In this study, a number of *C. sublyratus* leaf samples from of various sources were analyzed for their plaunotol content using the two methods and the results were compared. It can be seen in Table 7 and Fig. 14 that the values of plaunotol content of various leaf samples determined by TLC-densitometry were very closed to those obtained from the GC method. In terms of precision, the three separate determinations of each leaf sample showed a very narrow value of standard deviation of its plaunotol content (Table 8). These results indicated that the accuracy and precision of the TLC-densitometric method in the determination of plaunotol in *C. sublyratus* leaves were reliable.

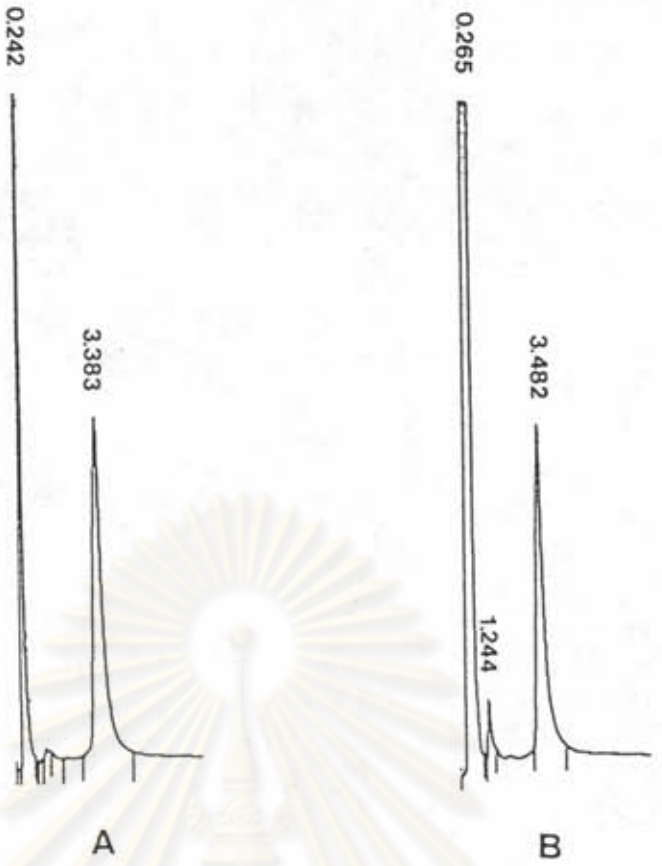

Figure 12 GC chromatogram of authentic plaunotol (A,RT = 3.383 min) and plaunotol in leaf extract (B,RT = 3.482 min).

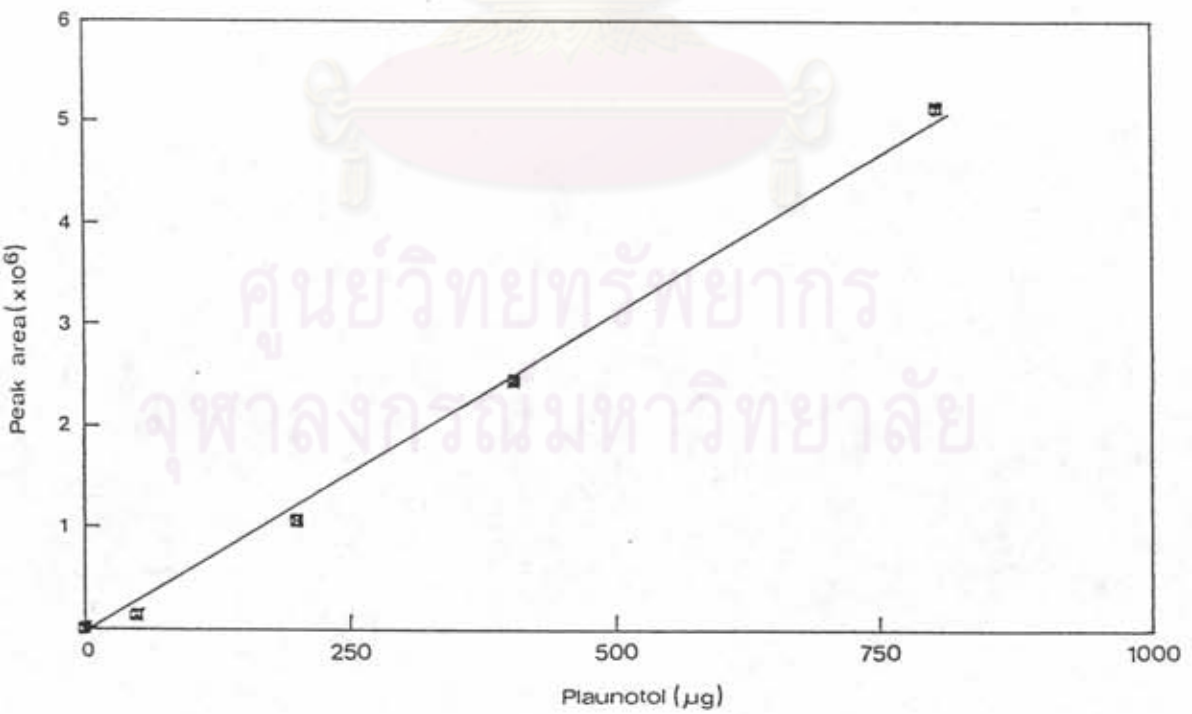

Figure 13 Calibration curve of plaunotol by GC method.

Table 7 Percentage of plaunotol content obtained from both TLC-densitometric and GC methods.

Sample	Plaunotol content (%w/w)	
	TLC-densitometric method	GC method
1	0.789±0.018	0.793±0.013
2	0.329±0.007	0.341±0.027
3	0.336±0.021	0.304±0.015
4	0.296±0.006	0.288±0.002
5	0.181±0.030	0.202±0.018
6	0.468±0.022	0.450±0.058
7	0.344±0.005	0.344±0.001
8	0.511±0.024	0.500±0.005

Each value represented the mean±SD of analysis in triplicate.

Samples 1-5 were obtained from the Faculty of Pharmaceutical Sciences(1), Faculty of Science (2 and 3) and the Nursery House (4 and 5) of Chulalongkorn University, Bangkok. Samples 6 and 7 were from Ban Singkhon, Prachuap Khiri Khan. Sample 8 were from the Medicinal Plant Garden, Mahidol University, Nakhon Pathom.

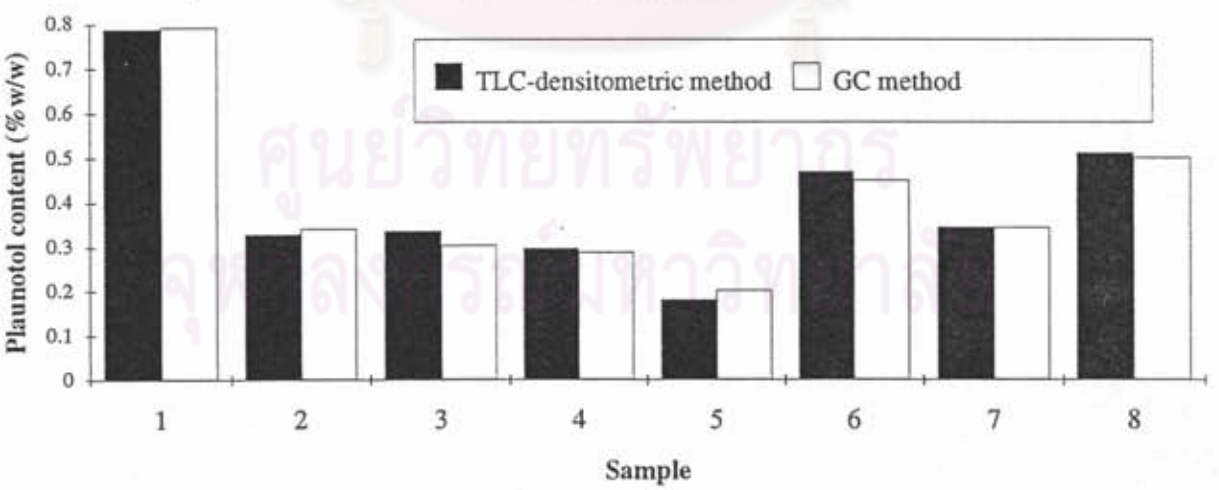

Figure 14 Bar graphs of plaunotol in *C. sublyratus* leaves determined by TLC-densitometric method (■) and GC method (❑). The values of plaunotol content were obtained from Table 7.

1.5 Sample Preparation for the Determination of Plaunotol in *C. sublyratus* leaves

A study on complete extraction of plaunotol from *C. sublyratus* leaves was carried out by comparing the efficiency of various extraction methods, including reflux, warm extraction, sonication and maceration. In this study, the dried ground leaves were extracted with 95% ethanol, the solvent usually used for plaunotol extraction (Sununta Cajesanun, 1991). After being extracted with various methods, plaunotol content in each ethanolic extract was quantitated by the TLC-densitometric method described in Section 1.1. The results are shown in Table 8. It was found that the method of reflux (80°C, 1 hour) gave the highest plaunotol content (0.330% dry weight) while the methods of warm extraction (70°C, 1 hour), sonication (50°C, 1 hour) and maceration (overnight) all gave lower plaunotol content (0.266%, 0.174% and 0.152% respectively). The extraction by reflux was therefore chosen for the quantitative extraction of plaunotol. For optimization of the ratio of dried ground leaves and 95% ethanol, it was found that the ratio of 200 mg sample in 10 ml 95% ethanol resulted in a complete extraction of plaunotol in a single reflux extraction since no plaunotol was detected in a subsequent extraction.

Table 8 Comparision of various methods for plaunotol extraction from *C. sublyratus* leaves.

Extraction method	Plaunotol content (%w/w)
Reflux (80°C, 1 hour)	0.330±0.020
Warm extraction (70°C, 1 hour)	0.266±0.015
Sonication (50°C, 1 hour)	0.174±0.024
Maceration (overnight)	0.152±0.003

2. Plaunotol content in *C. sublyratus* leaves

2.1 Variation of Plaunotol Content in *C. sublyratus* Growing in Various Parts of Thailand

Sixty-eight samples of *C. sublyratus* leaves were collected from various parts of Thailand, including Rayong, Prachuap Khiri Khan, Nakhon Pathom, Bangkok and Chachoengsao. After dryness, each sample was ground to powder and determined for its plaunotol content by TLC-densitometry established as described in Section 1.1. The results of plaunotol content in *C. sublyratus* leaves are summerized in Table 9 and Fig. 15. It can be seen that *C. sublyratus* leaves from various parts of Thailand contained highly variable plaunotol content ranging from 0.139 to 0.786% (w/w) dry weight (Fig. 15). The majority of the *C. sublyratus* leaf samples were found to contain plaunotol in the range 0.3-0.4% (w/w). The number of samples falling in this range were 27 equivalent to 39.7% of the collected samples. The next ranges were 0.4-0.5% (w/w) with 19 samples equivalent to 27.9% and 0.2-0.3% (w/w) with the frequency of 13.2% (Fig. 16 and Table 10).

When considering high plaunotol-producing *C. sublyratus* plants (more than 0.6% (w/w), it was found that samples PR4 from Rayong, PN2 from Nakhon Pathom and PB1 from Bangkok contained plaunotol in the level of 0.621, 0.757 and 0.786%, respectively. For low plaunotol-producing *C. sublyratus* plants, PR5 and PR8 from Rayong, PP18 from Prachuap Khiri Khan and PB23 from Bangkok were found to contain less than 0.2% (w/w) plaunotol in their dry leaves (Fig. 15).

2.2 Plaunotol Content in Mature and Young Leaves

C. sublyratus leaves from Prachuap Khiri Khan were obtained in three types including the old leaves from mature plants (PP1-PP8), young leaves from mature plants (PP9-PP16) and young leaves from young plants (PP17-PP24). All leaf samples were dried ground and determined for plaunotol content by TLC-densitometric method. As shown in Table 9 and Table 11, the average of total plaunotol content in the mature plants, pruning plants and growing plants were 0.425, 0.363 and 0.398% (w/w) dry weight, respectively. This results showed that three types of leaves had no significant difference in the content of plaunotol (α=0.05), although the mature leaves contained slightly higher plaunotol level than in the young leaves.

Table 9 Plaunotol content in *C.sublyratus* leaves obtained from various parts of Thailand.

Sample of *C.sublyratus* leaves	Plaunotol content (%w/w dry weight)
Rayong	
PR1	0.459±0.0310
PR2	0.348±0.0130
PR3	0.429±0.0069
PR4	0.621±0.0091
PR5	0.190±0.0165
PR6	0.441±0.0125
PR7	0.200±0.0086
PR8	0.139±0.0100
PR9	0.367±0.0163
Prachuap Khiri Khan	
PP1	0.583±0.0007
PP2	0.525±0.0119
PP3	0.402±0.0277
PP4	0.416±0.0310
PP5	0.364±0.0133
PP6	0.337±0.0201
PP7	0.320±0.0076
PP8	0.454±0.0057
PP9	0.283±0.0089
PP10	0.382±0.0249
PP11	0.329±0.0284
PP12	0.410±0.0173
PP13	0.468±0.0217
PP14	0.367±0.0112
PP15	0.344±0.0053
PP16	0.319±0.0175
PP17	0.413±0.0176
PP18	0.197±0.0047
PP19	0.396±0.0326
PP20	0.227±0.0134
PP21	0.505±0.0125
PP22	0.504±0.0203
PP23	0.491±0.0325
PP24	0.447±0.0314
Nakhon Pathom	
PN1	0.440±0.0269
PN2	0.757±0.0321

Table 9 (continued).

Sample of *C.sublyratus* leaves	Plaunotol content (%w/w dry weight)
Nakhon Pathom (continued)	
PN3	0.511±0.0235
PN4	0.440±0.0113
PN5	0.511±0.0163
Bangkok	
PB1	0.786±0.0176
PB2	0.235±0.0092
PB3	0.366±0.0110
PB4	0.452±0.0356
PB5	0.355±0.0058
PB6	0.329±0.0070
PB7	0.308±0.0180
PB8	0.431±0.0124
PB9	0.336±0.0214
PB10	0.336±0.0092
PB11	0.374±0.0035
PB12	0.206±0.0153
PB13	0.290±0.0036
PB14	0.296±0.0055
PB15	0.355±0.0180
PB16	0.359±0.0105
PB17	0.395±0.0092
PB18	0.476±0.0214
PB19	0.215±0.0095
PB20	0.360±0.0261
PB21	0.303±0.0184
PB22	0.402±0.0305
PB23	0.181±0.0301
Chachoengsao	
PC1	0.364±0.0301
PC2	0.338±0.0215
PC3	0.424±0.0075
PC4	0.412±0.0424
PC5	0.213±0.0279
PC6	0.351±0.0119
PC7	0.304±0.0229

The value of each sample represents the mean±SD of triplicate analysis. Samples PB1 and PB2 were obtained from the Faculty of Pharmaceutical Sciences, samples PB3–PB12 from the Faculty of Science and samples PB13–PB23 from the Nursery House of Chulalongkorn University.

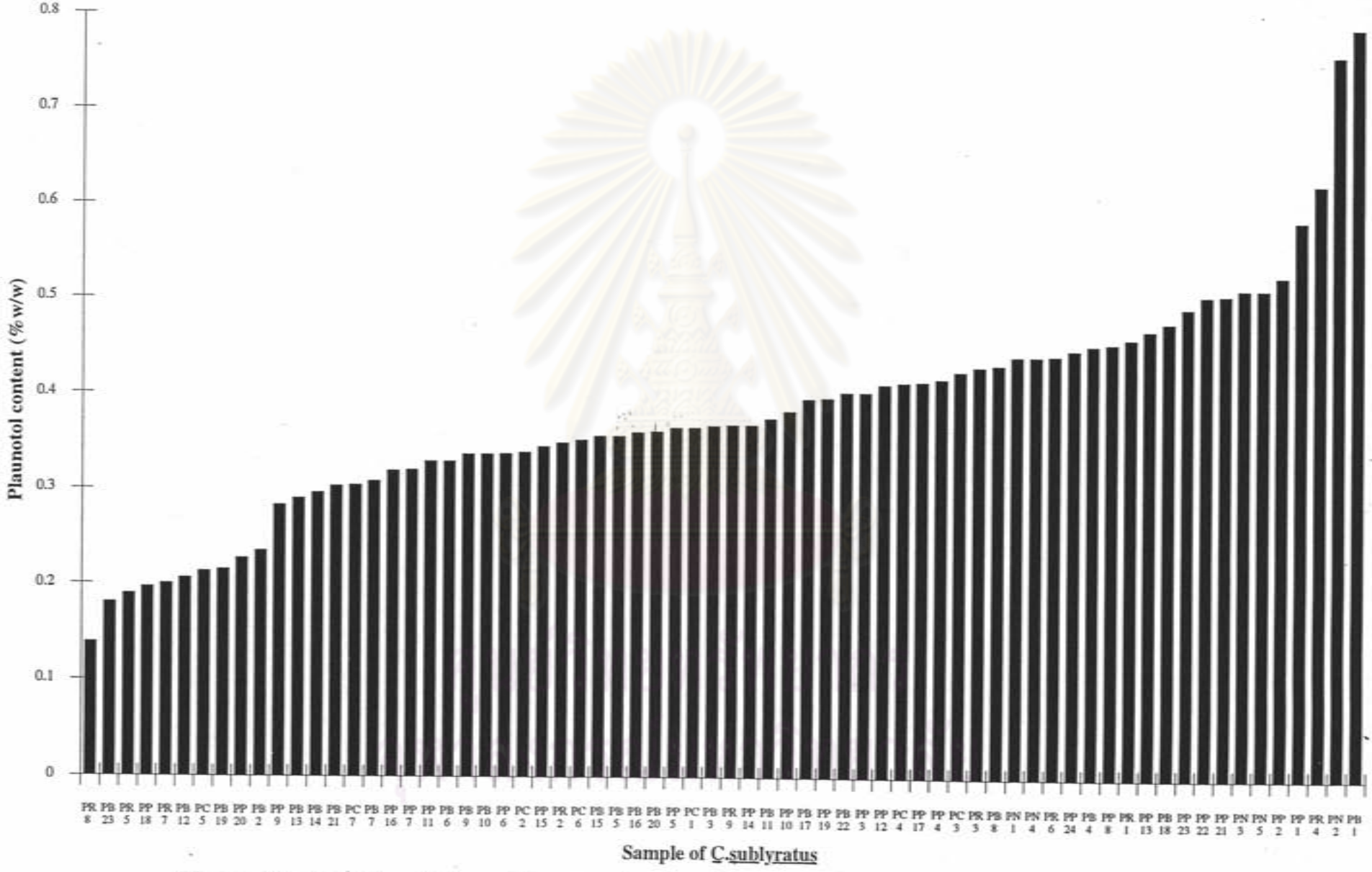

Figure 15 Variation of plaunotol content in *C. sublyratus* leaves obtained from various parts of Thailand.

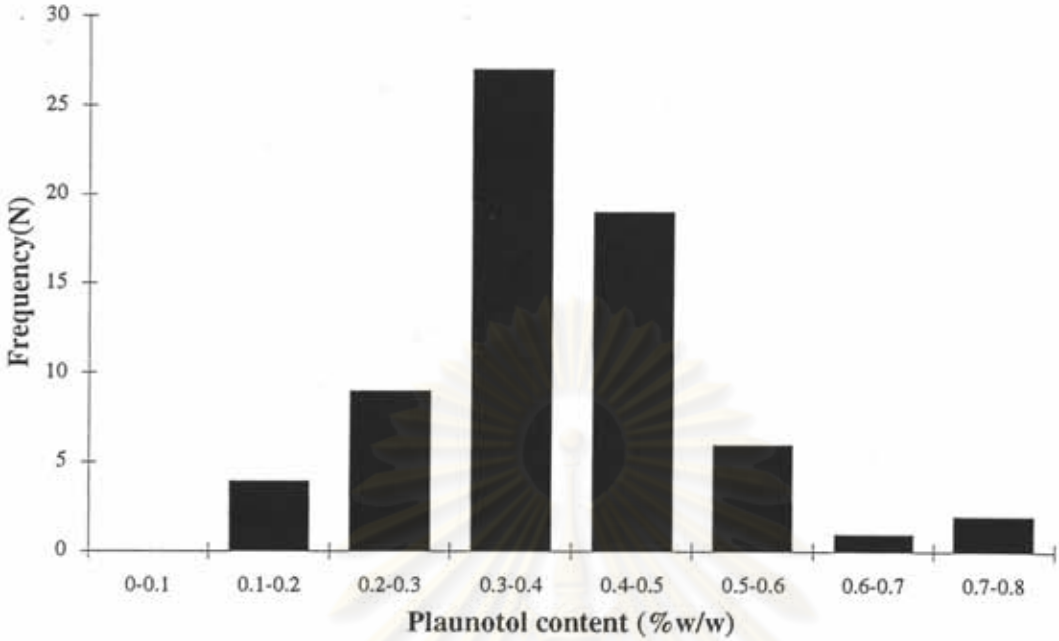

Figure 16 Distribution of plaunotol content in *C. sublyratus* growing in various parts of Thailand.

Table 10 The frequency of plaunotol content in *C. sublyratus* leaves.

Plaunotol content range (%w/w dry weight)	Frequency (N)	Percent (%)
0–0.1	0	0
0.1–0.2	4	5.88
0.2–0.3	9	13.24
0.3–0.4	27	39.71
0.4–0.5	19	27.94
0.5–0.6	6	8.82
0.6–0.7	1	1.47
0.7–0.8	2	2.94
Total	68	100

Table 11 The average total plaunotol content in *C.sublyratus* leaves were obtained from the mature plants, pruning plants and growing plants.

Leaf type	Number of samples	Average total plaunotol content (%w/w)
Mature leaves from mature plants	8	$0.425^{a}\pm0.0917$
Young leaves from mature plants	8	$0.363^{a}\pm0.0579$
Young leaves from young plants	8	$0.398^{a}\pm0.1217$

All samples were collected from Ban Singkhon, Amphor Muang, Prachuap Khiri Khan.

3. Detection of Plaunotol in Callus and Cell Suspension Cultures of *C. sublyratus*

The callus and cell suspension cultures of *C. sublyratus* were established as described in the Section 5.2 (page 67) and 5.3 (page 69). After a few passages of the callus and cell suspension cultures, they were examined for their ability to produce plaunotol. Concentrated ethanolic extracts of both culture types were prepared and their chemical constituents were separated by thin layer chromatography. Also, their TLC-densitometric chromatograms were produced by using TLC densitometer. In these experiments, the ethanolic extracts of the whole leaves were run parallel for comparison. The results of the TLC pattern and TLC-densitometric chromatogram are shown in Fig. 17. In Fig 17A, it clearly showed that plaunotol was not detected in the ethanolic extracts of the callus and cell suspension cultures of *C. sublyratus*. On the other hand, plaunotol was detected in the leaves of mother plant. These results were confirmed by their TLC-densitometric chromatograms (Fig. 17B). The results are similar to other reports that have been found to produce little or no plaunotol in callus cultures (Morimoto and Murai, 1989) and cell suspension cultures (Kitaoka, Nagashima and Kamimura, 1989).

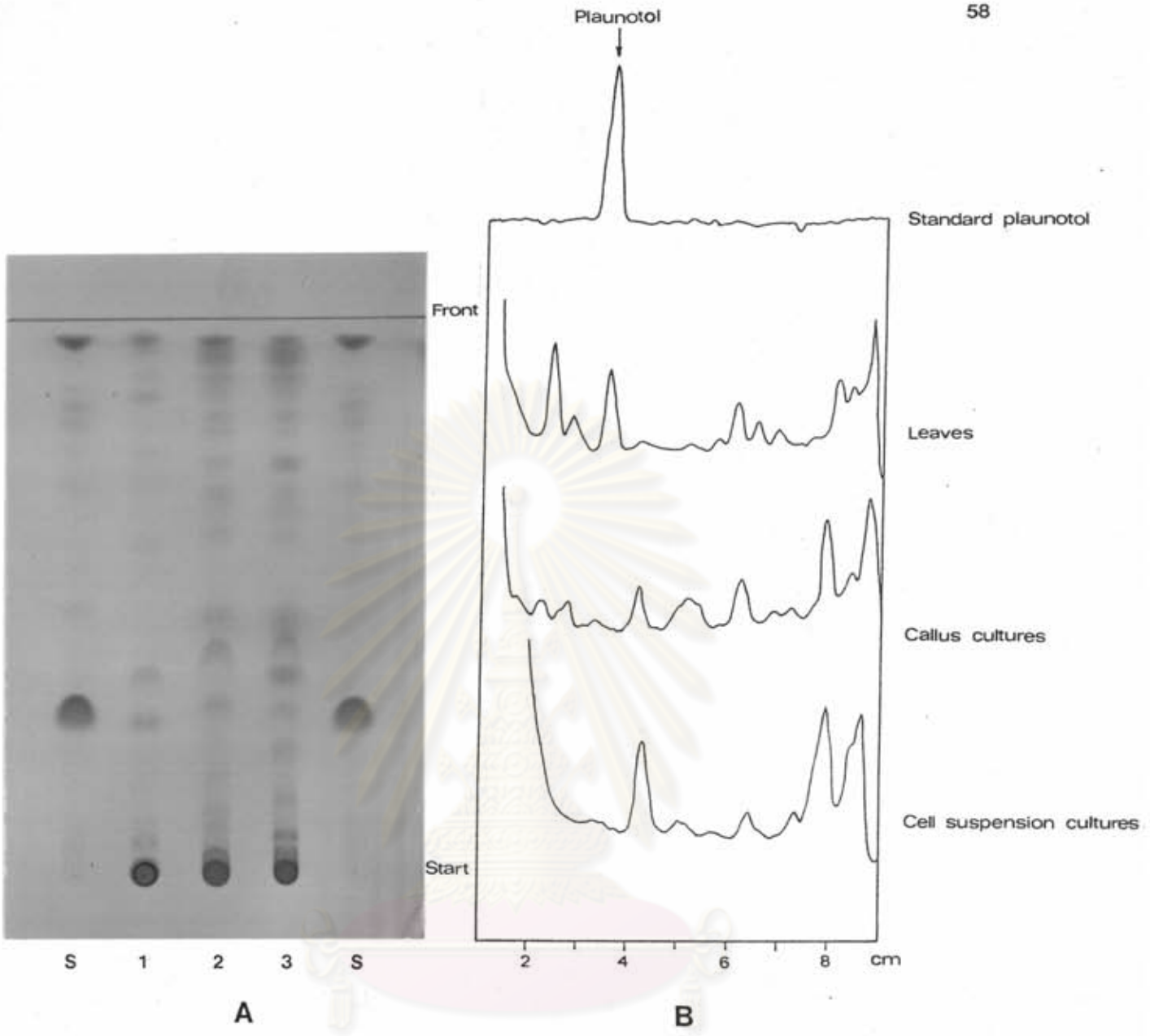

Figure 17 A) TLC patterns of the ethanolic extracts of leaves, callus and cell suspension cultures of *C. sublyratus*. S) authentic plaunotol, 1) leaves of mother plant, 2) callus cultures, 3) cell suspension cultures. TLC plate was detected for plaunotol by exposing with iodine.

B) TLC-densitometric chromatograms of the extracts of leaves, callus and cell suspension cultures of *C. sublyratus*.

4. Screening for Plaunotol-Containing Plants in Some *Croton* species

Because of its convenience and rapid performance, the developed TLC-densitometric method was used for screening other *Croton* plants that potentially contain plaunotol. In this study, seven species of genus *Croton*, including *C. sublyratus*, *C. oblongifolius*, *C. crassifolius*, *C. caudatus*, *C. tiglium*, *C. cascarilloides* and *Croton* sp. (Char plao nam ngoen) were tested. Their powdered materials were extracted with 95% ethanol under reflux and the extracts were detected directly for plaunotol by the TLC-densitometric method. The characteristic TLC patterns and TLC-densitometric chromatograms are shown in Fig. 18 and Fig. 19, respectively. In Fig. 18, it can be seen that the TLC patterns of various *Croton* plants after being exposed with iodine showed good separation of components. However, among these samples only *C. sublyratus* was found to contain plaunotol. Similary, the TLC-densitometric chromatograms in Fig. 18 also confirmed that no plaunotol was present in other *Croton* plants.

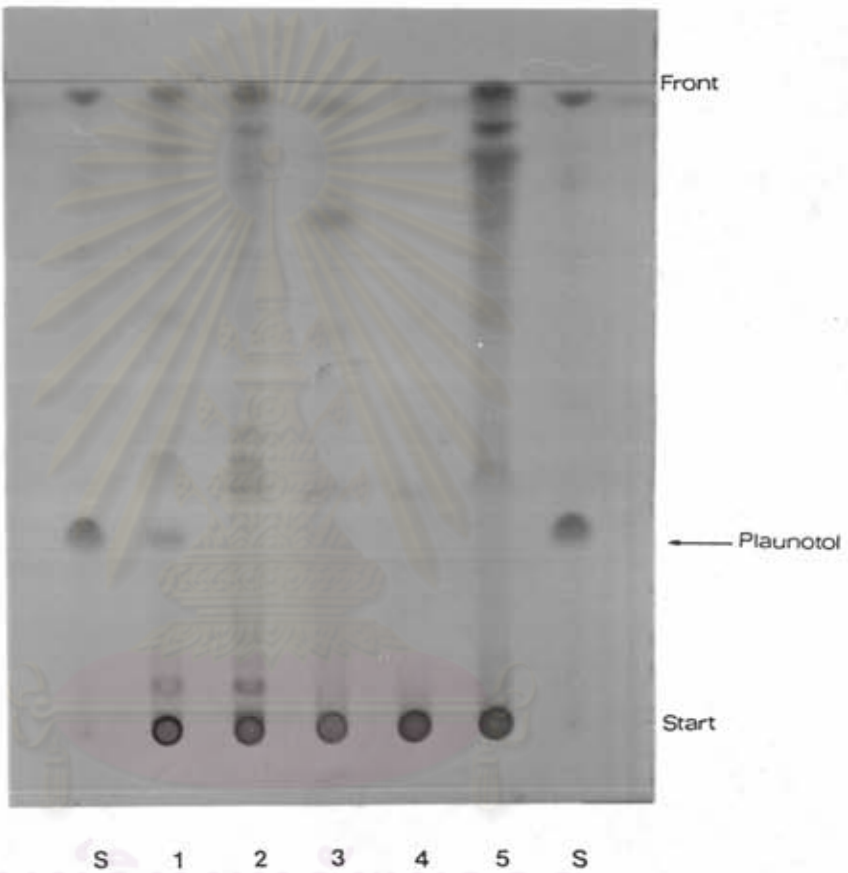

Figure 18 TLC patterns of the ethanolic extracts of various *Croton* plants. The patterns were observed after the TLC plate was exposed with iodine in a tank. S) authentic plaunotol, 1) *C. sublyratus* Kurz, 2) *C. oblongifolius* Roxb., 3) *C. crassifolius* Geisel, 4) *C. caudatus* Geisel, 5) *C. tiglium* Linn.

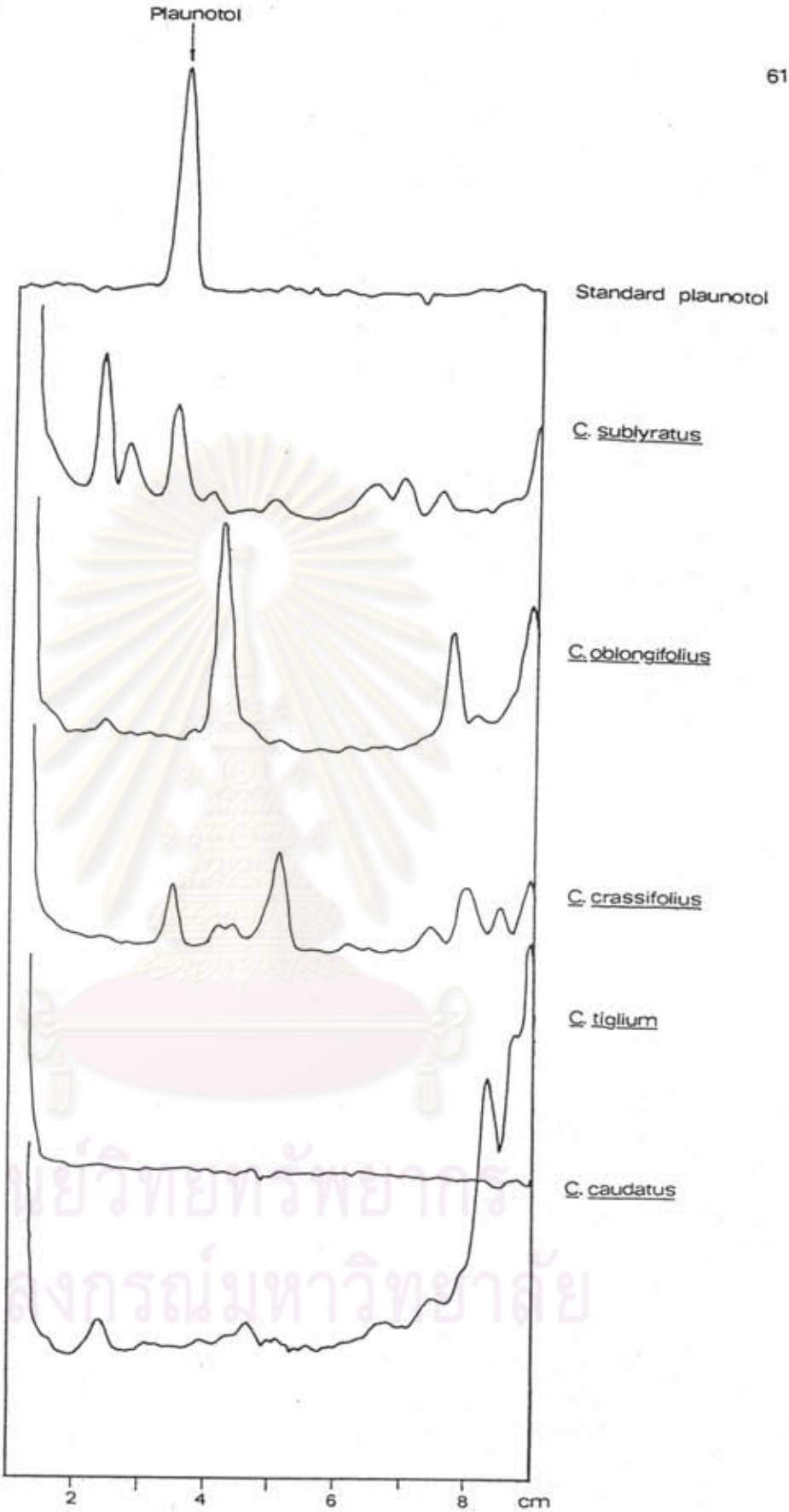

Figure 19 TLC-densitometric chromatograms of the ethanolic extracts of various *Croton* plants as compared to the chromatogram of standard plaunotol. The wavelength set for the scanning was 220 nm.

5. Tissue Cultures of *C. sublyratus*

5.1 Effects of Plant Growth Regulators on Growth and Development of Various Types of *C. sublyratus* Explants

5.1.1 Shoot Tips

Shoot tips of *C. sublyratus* were first chosen as explants for studying shoot formation of the plant. It was found that the shoot tips could initiate shoots on MS agar medium containing 2.0 mg/l BA. In this medium, the shoots were formed within 5 days followed by the appearence of new leaves. The elongation of the shoots and expansion of the leaves were continued for 2 weeks. Simutaneously, calli were formed at the base of petioles with fluffy texture and white color (Fig. 20). After culturing for 6 weeks, the developed leaves turned yellow and wilted, while the shoots turned brown and died.

By varying the concentration and type of auxins (IAA, IBA, NAA and 2,4-D), cytokinins (kinetin, BA and 2iP) and gibberellic acid (GA_3), it was found that the shoot tips developed in the same way as described above. No additional shoots were induced by all the tested media. When the shoot tips were cultured for 4 weeks, their growth and development were inhibited and the shoots turned yellow on the MS medium containing high concentration of IAA, IBA, NAA, kinetin, BA, 2iP, GA_3 (10 mg/l) or 2,4-D.

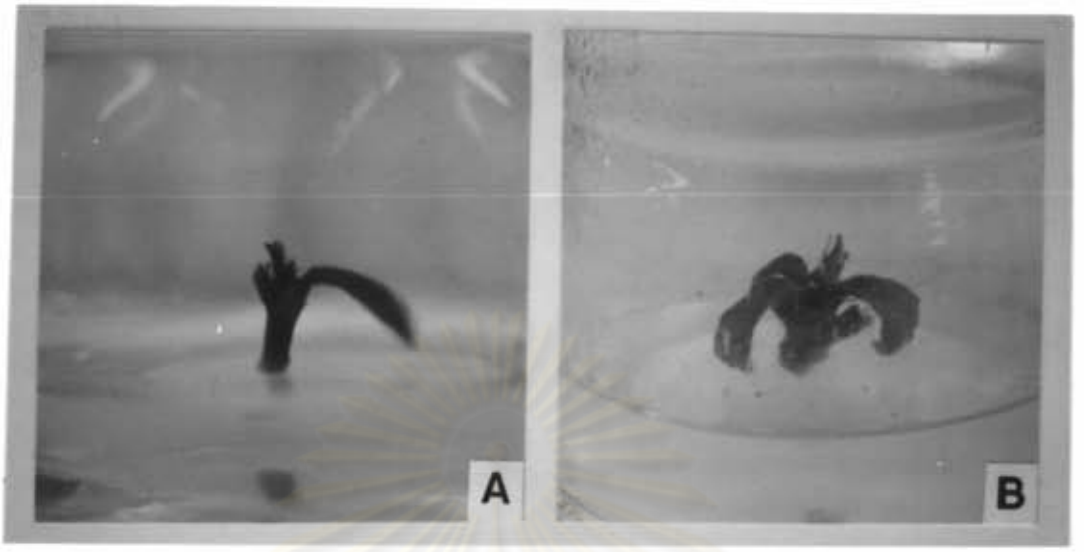

Figure 20 A) the shoot tip explant and B) the developed shoot tip on MS agar medium containing 2.0 mg/l BA.

5.1.2 Nodal Segments with Auxially Buds

When nodal segments with auxially buds were put onto MS agar medium containing 2.0 mg/l BA, the auxially buds appeared to develop to form shoots or calli. Apparently, three forms of the development from the auxially buds were observed :

First, the auxially bud developed to form shoot which subsequently developed futher to from leaves (Fig. 21A).

Second, the auxially bud developed to form shoot which did not grow and died eventually (Fig. 21B).

Third, the auxially bud did not form shoot (Fig. 21C).

However, in all types, calli were formed and appeared to be fluffy with white color at the around surface of node segment in the initiation stage and subsequently turned brown.

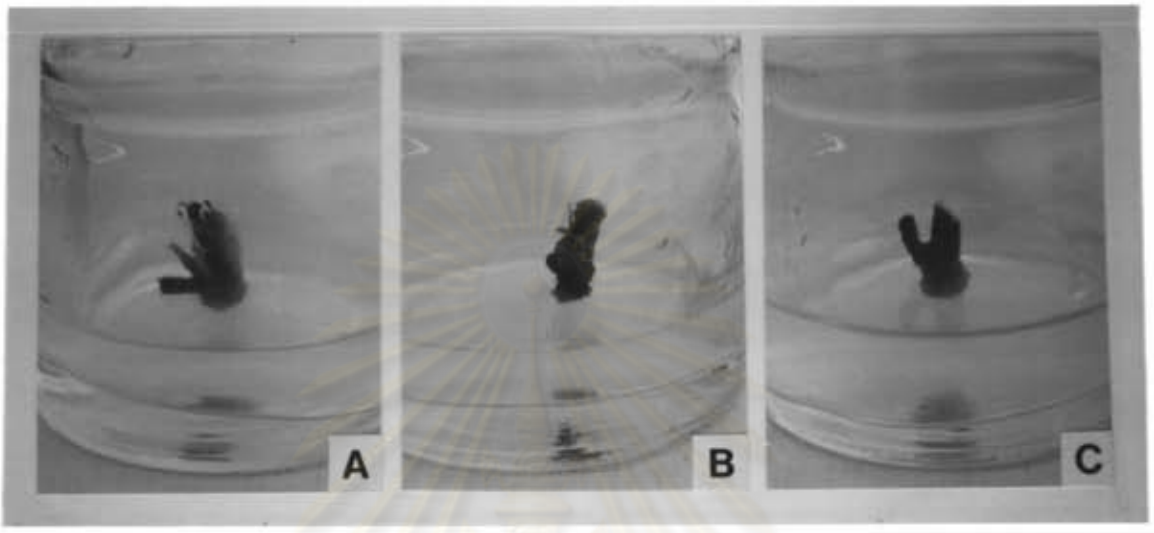

Figure 21 Three forms of the growth and development of auxially buds near by the node segment cultured on MS agar medium containing 2.0 mg/l BA. A) developed shoot, B) non-developed shoot and C) the auxially bud with no shoot formation.

5.1.3 Auxially Buds

Auxially buds dissected form nodal segments were found to form shoot on MS agar medium containing 2.0 mg/l BA (Fig. 22). Preliminary study showed that the age and size of the bud were important for the development of shoot. It was found that the shoot formation from mature buds was more effective than the small yound buds. However, the developed shoot appeared to be weak. The new leaves grew slowly and finally turned brown and died (Fig. 22 A to C).

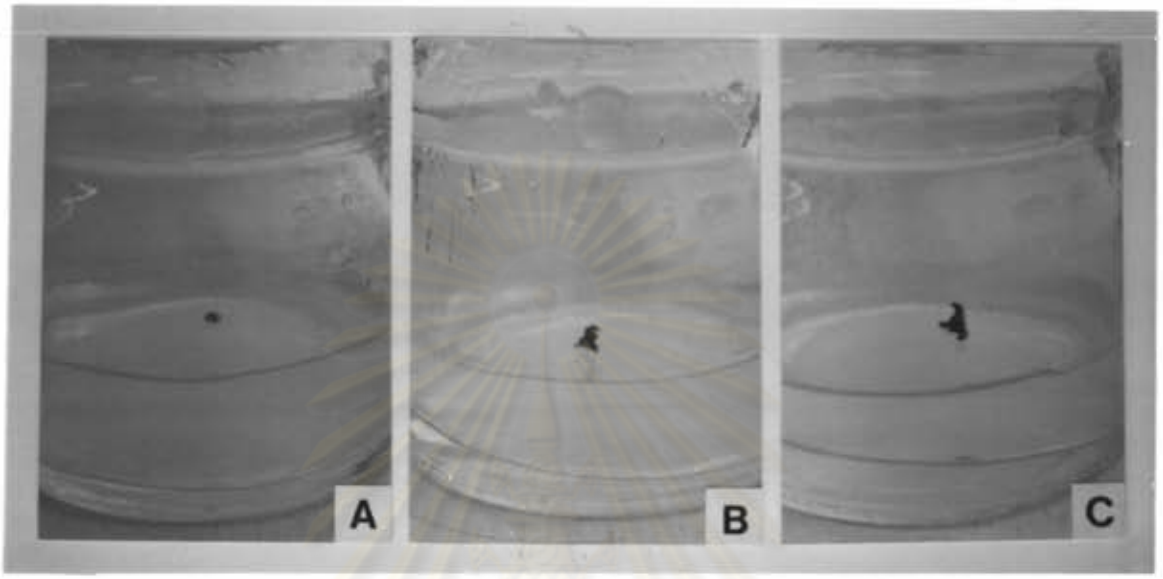

Figure 22 Steps of the shoot development from an auxially bud on MS agar medium containing 2.0 mg/l BA. A) an explant of auxially bud, B) developed shoot, C) dying shoot.

To check whether the shoot formation from the auxially bud was the results of internal factors of the bud or the hormonal factor in the medium, the bud explants were exposed with various conditions (Table 12). After three weeks, the buds on MS medium without growth regulators, 0.8% (w/v) agar medium or wet filter papers did not form shoot, while the buds on MS medium with BA formed shoot and leaves. In B5 medium with 2,4-D and BA, the bud was found to grow slowly and did not form shoot.

Table 12 Effect of various conditions for the shoot formation from auxially bud. Five buds were used for each treatment.

Condition	Growth and development of auxiallty buds
MS without growth regulator	No growth, the buds turned brown and died
MS+BA (2.0 mg/l)	Buds formed shoots and leaves
B5+2,4-D (0.5 mg/l)+BA (1.0 mg/l)	Buds grew slowly and did not form shoots
0.8% (w/v) Agar	No growth, the buds turned brown and died
Wet filter paper	No growth, the buds turned brown and died

5.1.4 Leaf Segments

The ability of the young leaf explants (0.5 x 0.5 cm^2) of *C. sublyratus* to undergo organogenesis was studied by plant growth regulators in MS and B5 media. By varying the concentration and type of auxins (IAA, IBA, NAA and 2,4-D), cytokinins (kinetin, BA and 2iP) and gibberellic acid (GA_3), it was found that the size of the leaf explants were highly increased in both media containing low concentration (0.01, 0.1 and 1.0 mg/l) of IAA, IBA, NAA, kinetin, BA, 2iP and GA_3. On the other hand, no increase in the explant size was observed in both media which contained high concentration (10.0 mg/l) of all the growth regulators. In MS medium supplemented with 2,4-D, the leaves appeared to roll and a little callus were formed at the edge of leaves. After culturing for a period of time, the leaves in all conditions turned yellow and no organogenesis was observed.

5.2 Estabishment of *C. sublyratus* Callus Cultures

In a preliminary experiment, the effect of basal media on callus induction from *C. sublyratus* explants was studied. The explants including young leaf and petiole were cultured on MS and B5 agar media containing 1.0 mg/l 2,4-D and 0.1 mg/l kinetin at 25±2°C under continuous light. Under these conditions, the ability of the young leaf and petiole explants to form callus was found to be different. While both MS and B5 media could induce callus formation from the leaf explant, no callus was observed with petiole explant under the same conditions. With the leaf explants, however, the developed calli appeared to grow more rapidly on MS medium than B5 medium. From these results, the leaf explants and the basal MS medium were chosen for subsequent studies. Firstly, the effect of the type and concentration of growth regulators on callus formation was investigated. It was found that callus formation was induced successfully on MS agar medium supplemented with the combination of 2,4-D (from 1.0 to 2.0 mg/l) and kinetin (from 0.5 to 1.0 mg/l). Especially, the combination of 2.0 mg/l 2,4-D and 1.0 mg/l kinetin appeared to be the best for callus formation. As shown in Fig. 23, the calli were initiated at the edge of leaf segments and formed as compact yellowish nodules after 2 weeks of incubation. The induced calli were subcultured successfully in every 3 weeks on MS agar medium containing 30 g/l sucrose, 1.0 mg/l 2,4-D and 1.0 mg/l BA. In this medium the callus had a high growth rate resulting in friable, soft and yellowish tissues (Fig. 24).

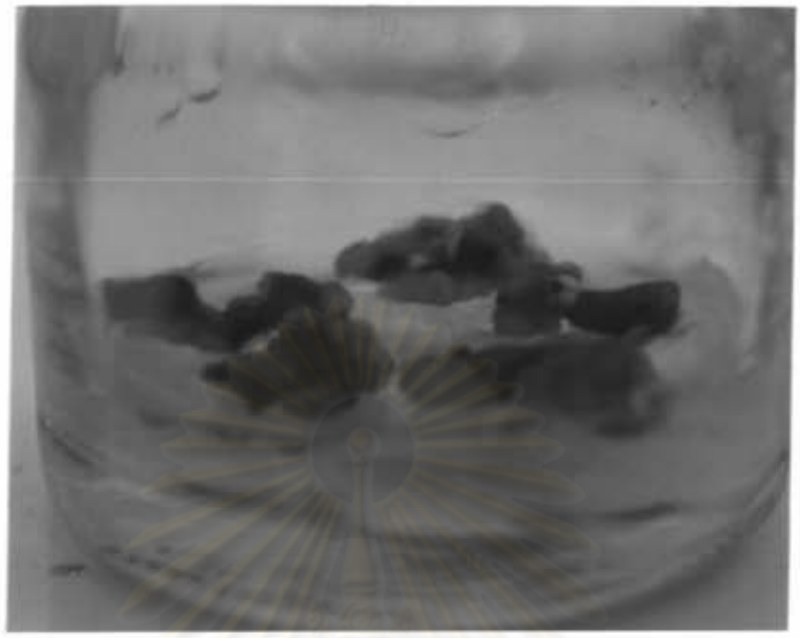

Figure 23 Induction of callus from the leaf explants of *C. sublyratus* on MS agar medium containing 2.0 mg/l 2,4-D and 1.0 mg/l kinetin.

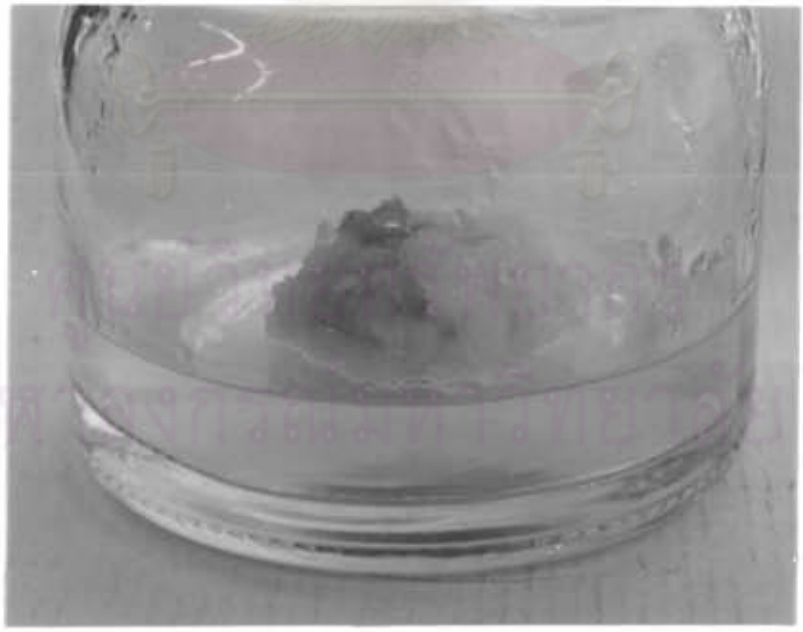

Figure 24 Callus cultures of *C. sublyratus* on MS agar medium containing 1.0 mg/l 2,4-D and 1.0 mg/l BA.

5.3 Establishment of *C. sublyratus* Cell Suspension Cultures

Cell suspension cultures of *C. sublyratus* were obtained from the friable yellowish callus which were maintained by a regular subculturing as described earlier. The callus tissues were separated into small aggregates before tranferring into MS liquid medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D and rotated at 120 r.p.m. on a rotary shaker. The cultured cells under these conditions formed small yellowish aggregates (Fig. 25). After stable cell suspension cultures were obtained, it was maintained in the same medium by subculturing every 4 weeks.

Figure 25 Cell suspension cultures of *C. sublyratus* maintained in MS liquid medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D.

5.4 Shoot Regeneration from *C. sublyratus* Callus Cultures

The establishment of adventitious shoots was found to the successful by starting from the callus cultures of *C. sublyratus* which had originally been established from the young leaf segments. As mentioned earlier (Section 5), the optimun conditions for callus formation were observed on MS agar medium containing 30 g/l sucrose, 2.0 mg/l 2,4-D and 1.0 mg/l kinetin. The characteristic tissues formed on this medium were compact yellowish callus formed at the cut of the leaf (Fig. 26A). After the callus grew to a bigger size (approximately 4 weeks), it was transferred onto MS medium containing 10 g/l sucrose, 2.0 mg/l NAA, 2.0 mg/l BA and 0.2% (w/v) gellan gum. The compact yellowish callus turned green and the cultures were maintained by regular transferring the callus onto the same medium for proliferation for every 3 weeks. It appeared that the growth of the callus was slow and the tissue were friable with green-yellow color (Fig. 26B). After 12 weeks, the callus was transferred onto MS medium containing 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 and 0.2% (w/v) gellan gum. Under these conditions, regeneration of adventitious shoots from the callus were observed after transferring the callus onto this medium for 6 weeks (Fig. 26C).

The detail of each step leading to the shoot regeneration is as follows :

Initially, the green-yellow callus (Fig. 27A) was maintained under the dark conditions for a week. During this period, the callus grew fast and formed fluffy with yellowish tissues (Fig. 27B). When exposed with light, the culture grew slowly but turned to green callus (Fig. 27C). After 4-6

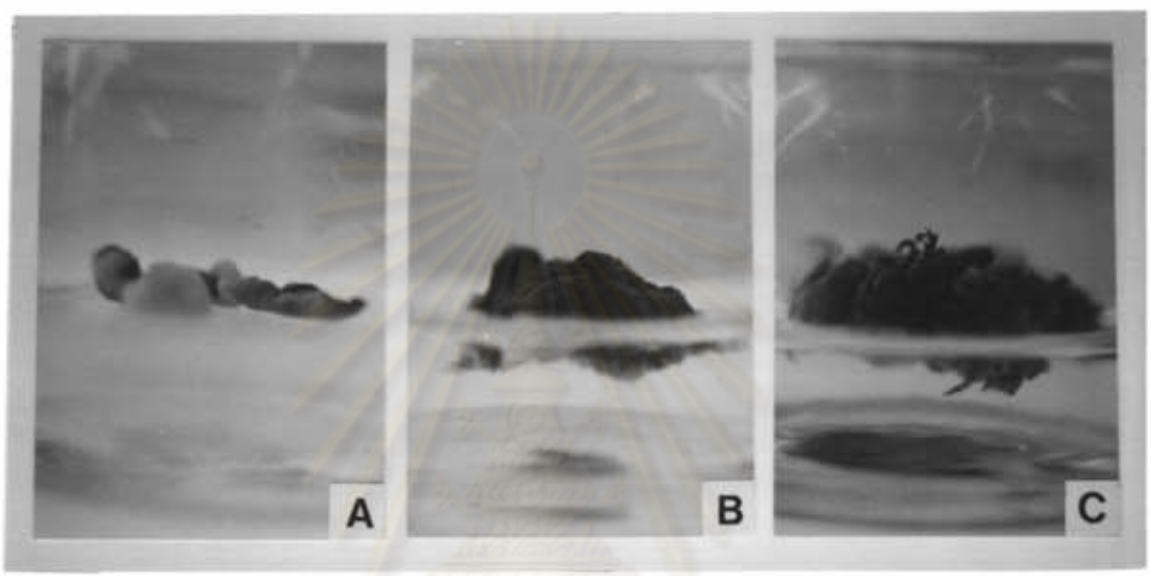

Figure 26 Steps of shoot regeneration from callus cultures of *C. sublyratus.*

A) : Callus induction from leaf segments on MS medium containing 30 g/l sucrose, 2.0 mg/l 2,4-D, 1.0 mg/l kinetin and 0.8% (w/v) agar.

B) : Callus cultured on MS medium containing 10 g/l sucrose, 2.0 mg/l NAA, 2.0 mg/l BA and 0.2% (w/v) gellan gum and formed green-yellow color and mass proliferation.

C) : Multiple adventitious shoots regenerated from green callus which grew on MS medium containing 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 and 0.2% (w/v) gellan gum.

weeks of the incubation, the green callus showed many green spots which induced adventitious shoots. The multiple shoot formation was found to increase after repeating transferring to fresh medium. The characteristic of multiple shoots is shown in Fig. 27D. It can be seen that the shoots formed short internodal length and swelled. After a long period of subculturing, the growth and development of the shoots were observed with the elongation of internode and formation of new leaves (Fig. 27E). After 21 weeks, the cultures formed brown callus with no friability. The shoots were separated and cultured on fresh medium.

The efficiency of the formation of adventitious shoots was 87.5% and the average number of shoots formed was 12.8 per callus (These results were obtained from the average of duplicate experiments for 21 weeks incubation).

From these results, it can be seen that the MS medium containing 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 and 0.2% (w/v) gellan gum could induce shoots from callus of *C. sublyratus* (the shoot formation medium). Subsequently, we further studied the medium composition for shoot regeneration, as described below.

The effect of agar on shoot formation. In this study, the green callus was transferred onto the shoot formation medium containing 0.6% (w/v) agar. The differentiation of the callus to form shoots on the agar medium was compared with the callus differentiation on the gellan gum medium. As shown in Fig. 28, the callus cultures on both the agar and gellan gum medium could formed multiple shoots. However, the greening of callus appeared to be reduced on the agar medium and their shoot induction

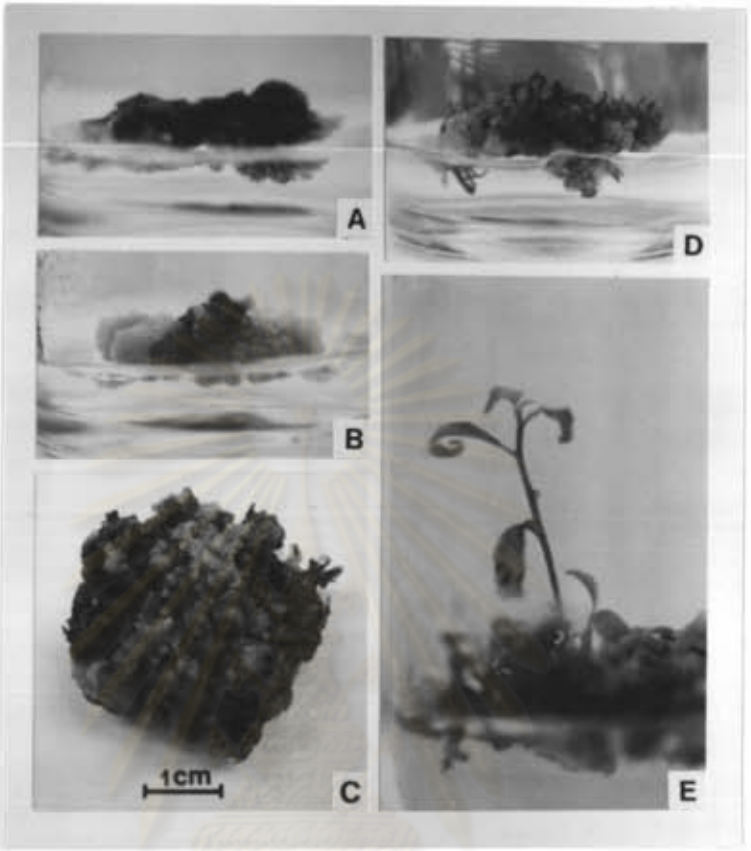

Figure 27 The growth and development of adventitious shoot from callus cultured on MS medium containing 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 and 0.2% (w/v) gellan gum at $25\pm2^{\circ}C$.

A) : Friable with green-yellow callus was placed on this medium for initial shoots.

B) : Fluffy with yellowish callus formed under the dark for a week.

C) : Friable, compact with green callus appeared under 16-hr photoperiod of fluorescent light at $25\pm2^{\circ}C$. It showed green spots which differentiated shoots.

D) : Multiple adventitious shoots were developed on the callus for 16 weeks.

E) : Shoot grew on this medium after 21 weeks incubation.

was slower than on the gellan gum medium. The percentage of shoot induction from the callus was found to be 66.7% on the agar medium and 87.5% on the gellan gum medium (Table 13). The number of shoots on the agar medium, however, was found to be much less than that on the gellan gum medium (Table 13). These results suggested that agar was not as good as gellan gum in the induction of multiple shoot formation from *C. sublyratus* callus.

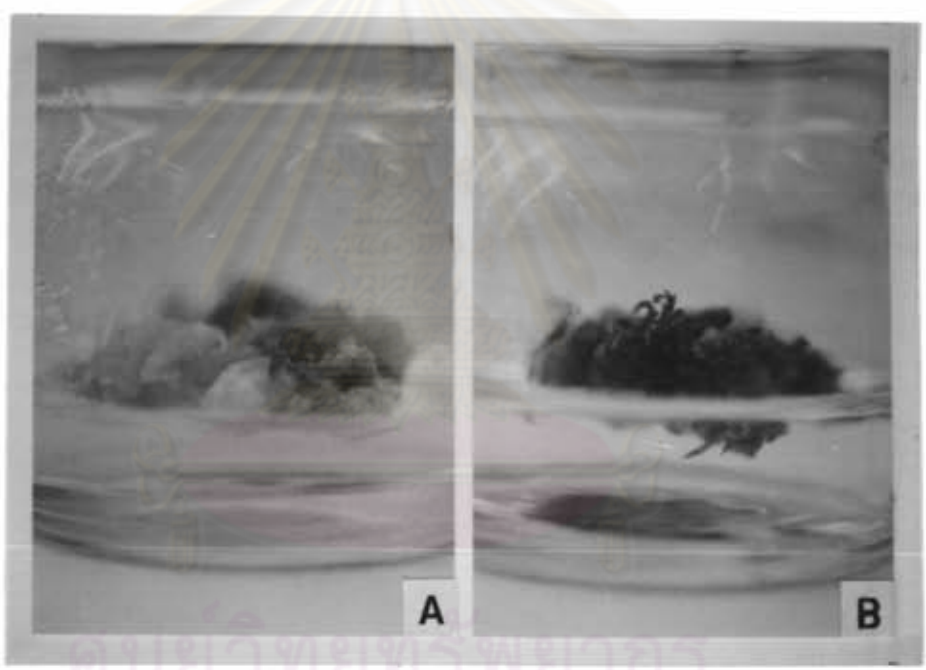

Figure 28 Shoot formation from callus cultures on MS medium containing 10 g/l sucrose, 1.0 mg/l BA and 0.05 mg/l GA_3 solidified with 0.6% (w/v) agar (A) and 0.2% (w/v) gellan gum (B) under 16-hr photoperiod of fluorescent light at $25\pm2^{\circ}C$ for 16 weeks.

Table 13 Effect of agar on multiple shoot formation of *C. sublyratus* callus. Callus cultures were mentained on MS medium containing 10 mg/l sucrose, 2.0 mg/l BA, 2.0 mg/l NAA and 0.2% (w/v) gellan gum for 16 weeks and transferred on MS medium containing 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 under 16-hr photoperoid of fluorescent light at 25±2°C for 20 weeks.

Gelling agent	No. of cultures examd.	% of shoot formation	No. of shoots/ culture
Agar	9	66.7	2.00
Gellan gum	8	87.5	18.14

In addition to the compact callus, the friable calli which were regularly subcultured on MS agar medium containing 30 g/l sucrose, 1.0 mg/l 2,4-D and 1.0 mg/l BA were also transferred onto the shoot formation medium containing 0.6% (w/v) agar and 0.2% (w/v) gellan gum medium. It was found that the friable, yellowish callus turned to be yellow-green callus both on the agar and gellan gum. The formed green callus on the agar medium was found to be less than on the gellan gum medium (Fig. 29). However, the shoot formation from the callus was not observed on these two media.

The effect of increased concentration of BA and GA_3 on shoot formation. MS medium containing 10 g/l sucrose, 2.0 mg/l BA and 0.5 mg/l GA_3 solidified with either 0.6% (w/v) agar or 0.2% (w/v) gellan gum was studied for the effect on shoot formation from callus. The results obtained

from this medium were compared with those from the shoot induction medium. It was found that the callus could grew on the two media which were solidified with gellan gum whereas no growth was observed with the agar medium. Furthermore, the shoots could also be formed on the two gellan gum medium after 15 weeks of incubation. The percentage of shoot formation and number of shoots on two medium were 83.3% (12.9 shoots per callus) on MS medium containing 1.0 g/l BA and 0.05 mg/l GA_3 57.1% (8.5 shoots per callus) on MS medium containing 2.0 mg/l BA and 0.5 mg/l GA_3, respectively (Table 14).

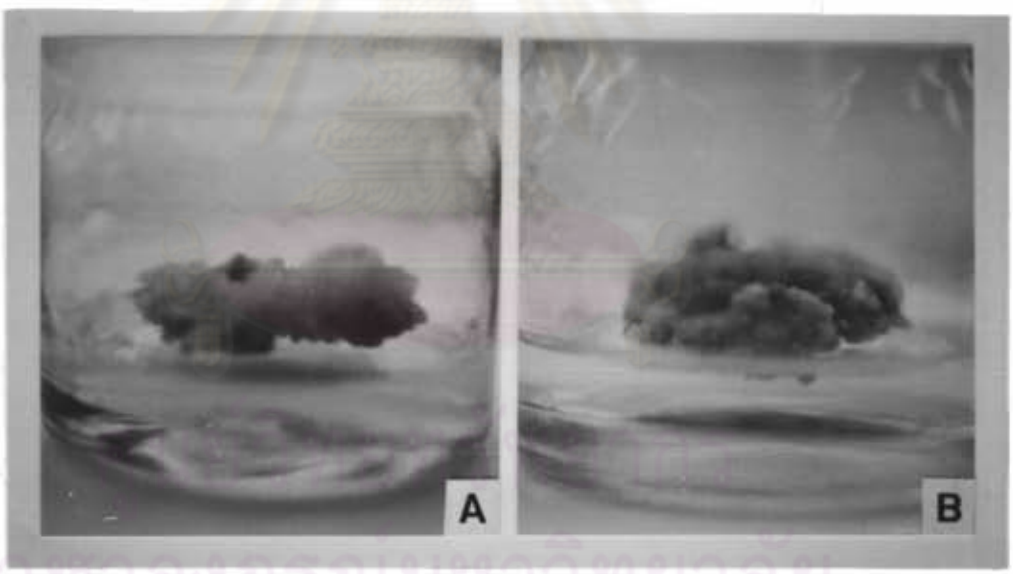

Figure 29 The growth and development of callus on MS agar medium containing 30 g/l sucrose, 1.0 mg/l 2,4-D and 1.0 mg/l BA and were transferred on MS medium containing 10 g/l sucrose, 1.0 mg/l BA and 0.05 mg/l GA3 solidified with 0.6%(w/v) agar (A) and 0.2%(w/v) gellan gum (B).

Table 14 Effect of BA and GA_3 concentrations on shoot formation of induced callus from leaves. Callus was induces from leaves on MS medium containing 30 g/l sucrose, 2.0 mg/l 2,4-D, 1.0 mg/l kinetin and 0.8% (w/v) ager for 6 weeks before being transferred onto various medium under 16-hr photoperoid of fluorescent light at $25\pm2^{\circ}C$ for 21 weeks.

Growth regulator (mg/l) and gelling agent on MS medium containing 10 g/l sucrose	% of shoot formation	No. of shoots/ culture
BA (1.0 mg/l)+GA_3 (0.05 mg/l)+0.2% gellan gum	83.3	12.9
BA (2.0 mg/l)+GA_3 (0.5 mg/l)+0.2% gellan gum	57.1	8.5
BA (2.0 mg/l)+GA_3 (0.5 mg/l)+0.6% agar	0	0

5.5 Root Induction

The induction of rooting from the differentiated shoots was studied by cutting the shoot and pot it onto a hormone-free MS medium (Fig. 30). It was found that shoots grew slowly and the leaves turned yellow-orange color with wilted after 2 weeks of incubation. The rooting was not induced by transferring the shoots on this medium.

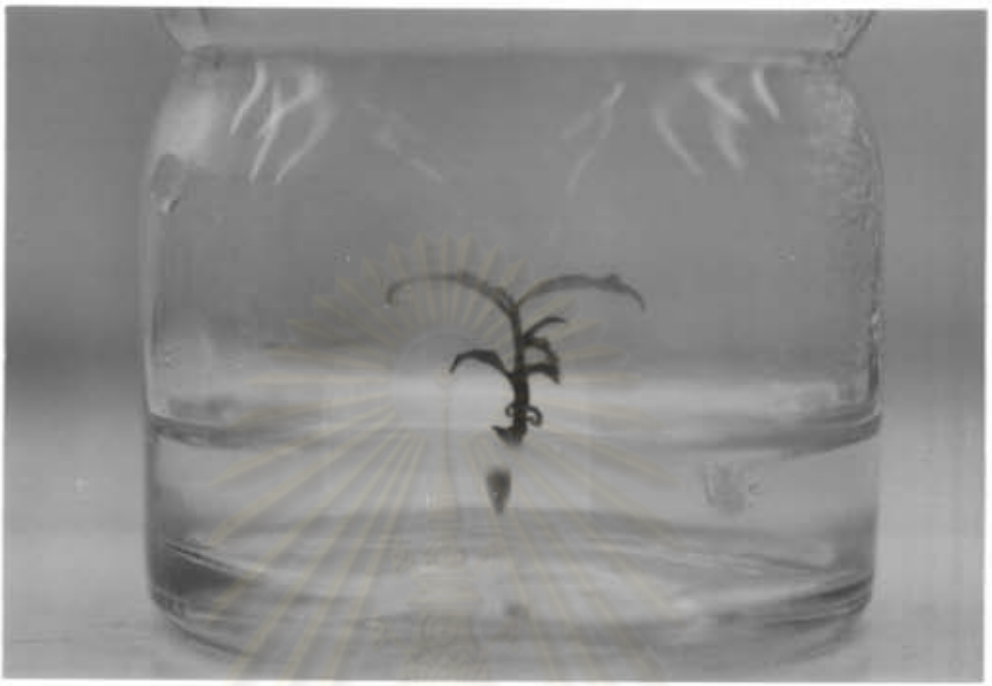

Figure 30 The shoot was cultured on hormone-free MS medium for root induction.