CHAPTER III

RESULTS

Callus initiation

1. Tissue Sterilization.

It is always necessary to remove all microorganisms from tissue to be cultured because they will outgrow the tissues and destroy them. Secondly, the presence of microorganisms changes the enveronment by removing nutrients from the medium and by releasing metabolic by-products into it. As shown in Table 7, a wide variation of between 3.3 to 100% contamination, 10 to 53.3% dead tissue and 0 to 100% survival percentage from the different treatments were observed.

The results could be grouped into four. The first group to comprises of treatments number A1 to A 6, A11 and A16 which was 100% contamination and no survival tissues was observed. The second group comprises of treatments number A14 and A15, A18 to A20 which had no contaminaton percentage. High survival tissues and also had some dead tissues were presented in this group. Out of the remaining 7 treatments, 0% comtamination and 100% survival was recorded in 2 treatments(number A9 and A10), and less than 25% contamination and more than 60% survival was recored in five treatments, A7 and A8, A12, A13 and A17.

It is also evident from the previous mention that there was an overall of 100% survival due to treatments number A9 and A10

Table 7 Apparent scores of sterilized pyrethrum leaf tissues after 7 days inoculation under selection treatments

Treatment No.	Percentage		
	Contamination	Dead tissue	Survival tissue
A1	100.0	0.0	0.0
A2	100.0	0.0	0.0
A3	100.0	0.0	0.0
A4	100.0	0.0	0.0
A5	100.0	0.0	0.0
A6	100.0	0.0	0.0
A7	22.0	0.0	78.0
A8	7.8	0.0	92.2
A9	0.0	0.0	100.0
A10	0.0	0.0	100.0
A11	100.0	0.0	0.0
A12	23.3	0.0	76.6
A13	3.3	0.0	96.7
A14	0.0	10.0	90.0
A15	0.0	26.5	73.5
A16	100.0	0.0	0.0
A17	6.6	10.0	83.3
A18	0.0	36.6	63.3
A19	0.0	53.3	46.6
A20	0.0	43.3	56.6

All tissues were placed on full strength MS agarized medium containing 2,4-D (1.0 mg/l), BA (3.0 mg/l) under 2,000 lux of fluorescent illumination with an 8-hour dark period and maintained at a temperature range of 25 $\pm$ 2 °C

(group 3) which had ranked first in this study, followed by A8, A13 and A14 which had a survival percentage over 90.

2. Establishing callus culture

2.1 Medium formulation.

Different growth regulator systems were used for callus initiation and multiplication. The swelling of the leaf and petiole explants were observed within 10 days after inoculation and the callus initiation noted from 16th day onwards were shown in Figures 9 and 10. Successful initiation and establishment of friable callus on different media from the leaf and petiole explants were only achieved in few treatments with MS basal medium containing 2,4-D and BA combination. The results were summarized in Table 8, Figures 10 and 11.

Four groups of explant response results were defined for each charactors. The first group comprised less than 1.0 GI units, the range of 0.2 to 1.0 and 0.0 to 50.0 of score signifies intensity of callusing units and callusing percentage respectively. The second group, about 1.96 and 2.61 GI units, 1.98 and 2.30 callus intensity scoring unit and 30.0 and 36.6% callusing were recorded. The third group, wide variation of GI were observed in treatments number B13 to B 18 which had 2.2 to 4.0 GI unit and 33.3 to 56.6 callusing percentage, and about 2.0 average callus intensity scoring. The forth group obtained over 3.43 to 7.63 GI units, 68 to 91% callusing and approximately 3.0 scoring units. These of suitable treatments (group 4) were retested and the reconfirmed results were illustrated in Figure 12. The illustrations of treatment with MS basal medium containing 2,4-D and BA (1.0 : 3.0 mg/ml) provided the highest callusing percentage (91%),

Table 8 Influence of growth regulator (2,4-D + BA) on initiation and establishment of leaf-derived callus of pyrethrum

Treatment No.	2,4-D:BA	* Cullusing (%)	Score signifies size of callus	Initial wet weight (g)	Final wet weight (g)	** GI
B1	0.0:0.0	0.0	0.0	0.0167	0.0158	0.00
B2	0.0:1.0	0.0	0.0	0.0153	0.0152	0.00
B3	0.0:2.0	0.0	0.0	0.0162	0.0150	0.00
B4	0.0:3.0	0.0	0.0	0.0130	0.0180	0.00
B5	0.5:0.0	0.0	0.0	0.0250	0.0098	0.00
B6	0.5:1.0	30.0	0.0	0.0300	0.0420	0.38
B7	0.5:2.0	33.3	1.0	0.0380	0.0570	0.50
B8	0.5:3.0	50.0	1.0	0.0280	0.0380	0.36
B9	1.0:0.0	26.6	0.2	0.0280	0.0380	0.36
B 10	1.0:1.0	68.0	2.7	0.0210	0.0930	3.43
B 11	1.0:2.0	73.0	3.2	0.0187	0.1330	6.11
B 12	1.0:3.0	91.0	3.7	0.0176	0.1520	7.63
B 13	1.5:0.0	36.6	2.0	0.0360	0.1240	2.44
B 14	1.5:1.0	43.3	2.70	0.0290	0.0126	3.34
B 15	1.5:2.0	56.6	2.75	0.0200	0.1000	4.00
B 16	1.5:3.0	46.6	2.40	0.0230	0.0870	2.78
B 17	2.0:0.0	33.3	2.00	0.0250	0.0800	2.20
B 18	2.0:1.0	40.0	2.50	0.0230	0.0900	2.91
B 19	2.0:2.0	36.6	1.98	0.0270	0.0800	1.96
B 20	2.0:3.0	30.0	2.30	0.0230	0.0830	2.61

All explants were placed on full strength MS agarized medium containing various concentration of growth regulators under 2,000 lux of fluorescent illumination with an 8-hour dark period and maintained at a temperature range of 25 $\pm$ 2 °C

* (No. of callus tissue)(100)/No. of experimental tissue (30)
No response tissue (still remain green colour)

** Growth index = $\frac{F - I}{I}$

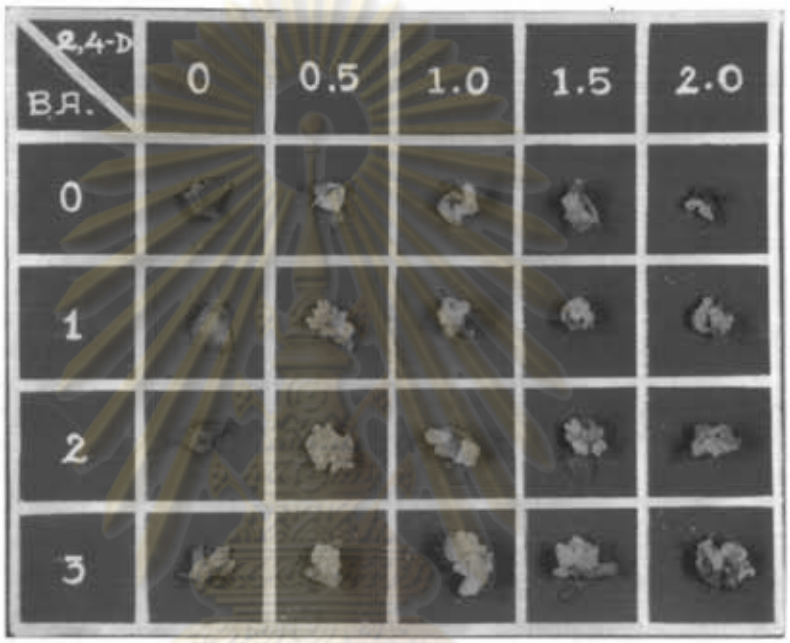


Figure 10 Effect of growth regulator (2,4-D and BA) at various levels on callus initiation from leaf which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25 ± 2 ^{o}C

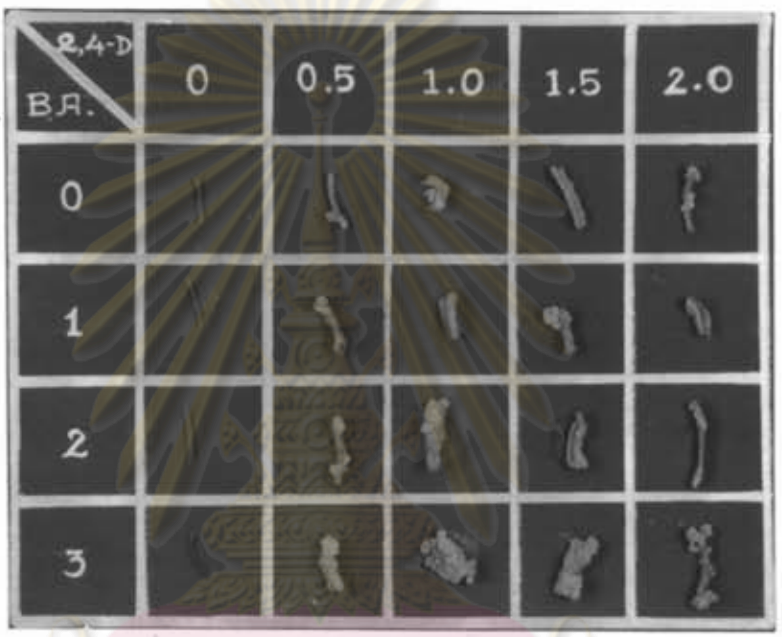


Figure 11 Effect of growth regulator (2,4-D and BA) at various levels on callus initiation from petiole which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temparature range of 25 ± 2 ^{o}C

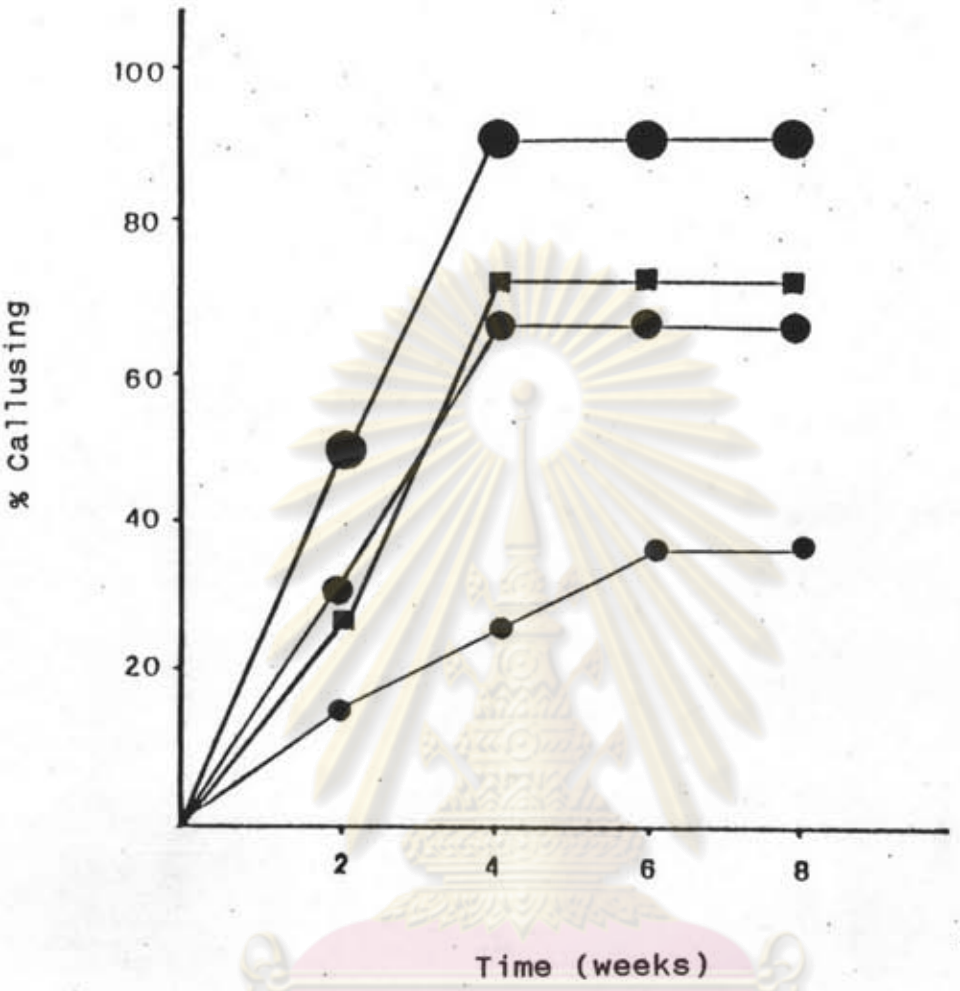


Figure 12 Effect of growth regulator (BA) at various levels recombined with 2,4-D (1.0 mg/l) on callus initiation of leaf blade which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25 ± 2°C

- 2,4-D and BA (1.0 : 0.0 mg/l)
- 2,4-D and BA (1.0 : 1.0 mg/l)
- 2,4-D and BA (1.0 : 2.0 mg/l)
- 2,4-D and BA (1.0 : 3.0 mg/l)

and yield of the desired friable callus.

Maximum callusing and proliferation were found in the range of 3 to 4 weeks after inoculation with that said medium. The specified treatment, B 12 yeilded the maximum GI on fresh weight basis (7.63) in 6 weeks of which declined during the 8th weeks (6.2). These callus cultures were subcultured over 15 times, affording the friable callus within the same media. For another one point, the comparative study of callus initiation in each source was also proved that callusing percentage were ratively equivalent in both leaf and petiole while the stem pieces obtained significant lower (Figure 13).

Salt requirment for culture callus was investigated on full and half strengths of medium formulation. The different growth rate and yield of proliferating cells were recorded and summarized results were shown in Table 9. Figures 14 indicate that half strength MS medium comprises a low growth rate that is less than half of another (20.38:38.75 GI units)

2.2 Environmental factors

2.2.1 Light

Leaf derive-calli was established by placing explants directly onto MS basal medium as given in the Material and Method section. The growth rate represented in term of GI, scoring of callusing of both groups had been recorded by harvesting 10 flasks each for 8 weeks starting with day 0. The results obtaining from data in Table 10 and Figures 15 revealed that GI of the culture as referred to Solid Dark grown culture was meagre (3.74) and hence the sign

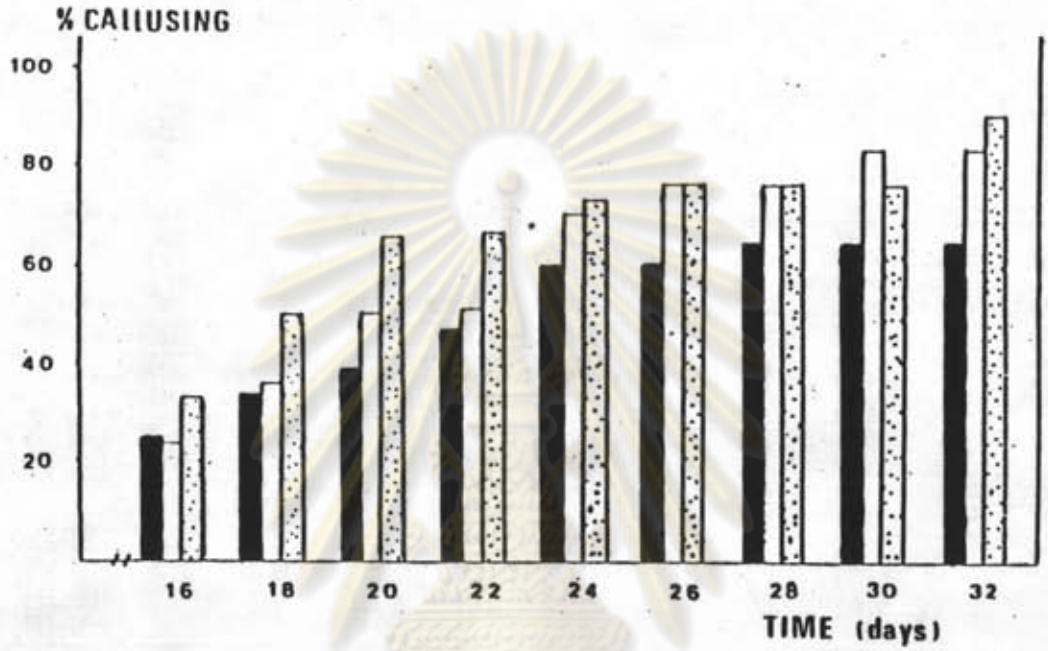


Figure 13 Effect of 2,4-D and BA (1.0:3.0 mg/l) on callus initiation of various organ sources which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25 $\pm$ 2^{o}C.

stem

leaf petiole

leaf blade

Table 9 Influence of salt concentration on establishment of leaf-derived callus of Pyrethrum

a) Half strength

No. of Replication	Score signifies size of callus	Initial wet weight (g)	Final wet weight (g)	* GI
1	2.0	0.0190	0.0910	3.79
2	3.0	0.0210	0.1340	5.38
3	2.0	0.0200	0.0780	2.90
4	3.0	0.0210	0.1380	5.57
5	3.0	0.0160	0.1320	7.25
6	3.0	0.0170	0.1130	5.65
7	2.0	0.0190	0.0790	3.10
8	4.0	0.0150	0.1370	2.13
9	3.0	0.0230	0.1180	4.13
10	2.0	0.0260	0.0910	2.50
X	2.7	0.0197	0.1111	4.24
SD	0.640	0.0031	0.0231	1.58
CV (%)	25.00	16.75	21.93	38.75

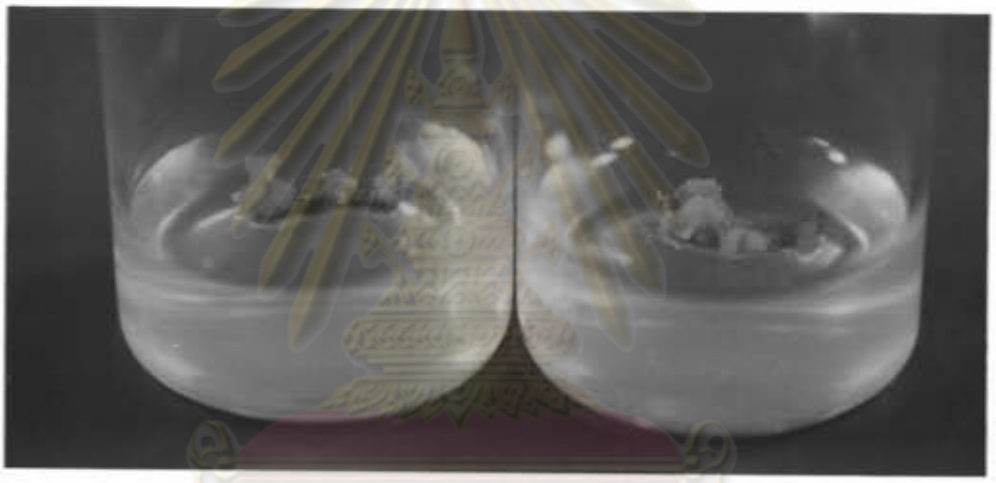
b) Full strength

No. of Replication	Score signifies size of callus	Initial wet weight (g)	Final wet weight (g)	* GI
1	4.0	0.0160	0.1400	7.75
2	3.0	0.0180	0.1320	6.33
3	3.0	0.0180	0.1180	5.56
4	4.0	0.0190	0.1720	8.05
5	3.0	0.0210	0.1170	4.57
6	4.0	0.0200	0.1690	7.45
7	3.0	0.0220	0.1130	5.05
8	4.0	0.0170	0.1500	7.82
9	4.0	0.0230	0.1800	6.83
10	3.0	0.0210	0.1270	5.05
X	3.5	0.0195	0.1418	6.45
SD	0.500	0.0022	0.0235	1.25
CV (%)	15.06	11.66	15.92	20.38

All explants were placed on half and full strength MS agarized medium containing 2,4-D (1.0 mg/l), BA (3.0 mg/l) under 2,000 lux of fluorescent illumination with an 8-hours dark period and maintained at a temperature range of 25 ± 2 °C

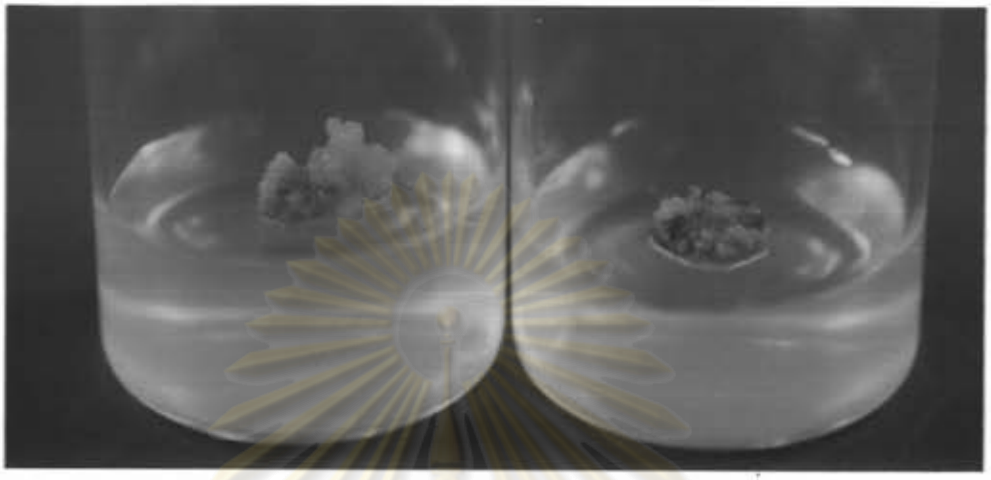
* Growth index = $\frac{F - I}{I}$

(A)



(B)

Figure 14 Effect of medium concentration, half strength (left) and full strength (right) on callus initiation of leaf (A) and petiole (B) which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25 ± 2 ^{o}C.



(A)

(L) (D)

(B)

(L) (D)

Figure 15 Effect of Light and dark period on callus initiation from leaf (A) and petiole (B) which had been cultured on MS basal medium and maintained at temperature range of 25 ± 2 °C.
L Light grown culture
D Dark grown culture

Table 10 Influence of light and dark period on the establishment of leaf-derived callus of Pyrethrum

Experimental conditions	Score signifies * size of callus	Initial wet weight (g) **	Final wet weight (g) **	GI ***	Relative percentage of GI
2,000 lux. 8 hr. dark period	3.85	0.016	0.14	7.75	100
Continuous dark	2.01	0.019	0.09	3.74	48.22

All explants were placed on full strength MS agarized medium suplemented with 2,4-D (1.0 mg/l), BA (3.0 mg/l) under selected illumination and maintained at a temperature range of 25 ± 2 °C

* 10 replecations
** average callus weights of 10 replicates
*** Growth index = $\frac{F - I}{I}$

Table 11 Effect of temperature on pyrethrum leaf-derived callus initiation

Experimental conditions (C)	Score signifies * size of callus	Initial wet weight (g) **	Final wet weight (g) **	GI ***	Relative percentage of GI
25	3.50	0.023	0.18	6.826	100
30	1.96	0.024	0.09	2.75	40.287

All explants were placed on full strength MS agarized medium suplemented with 2,4-D (1.0 mg/l), BA (3.0 mg/l) under selected illumination and maintained at a temperature range of 25 ± 2 °C

* 10 replecations
** average callus weights of 10 replicates
*** Growth index = $\frac{F - I}{I}$

signified intensity of callusing (2.01) was also found to be lower than the tissues grown in light, Solid Light grown culture.

Therefore, GI of these said cultures were re-evaluated for comparative study on the relative percentage corresponding to each other. Thus was found to comprise nearly two-fold increasing of growth rate when the culture were grown under photoperiodic condition.

2.2.2 Temperature

According to the result obtained from Table 11 which was evaluated by the mean value ($\bar{x}$) of 10 replicate analyses. It was evident that pyrethrum callus culture preferably grew at 25 $\pm$ 2^{o}C more than 30^{o}C. GI of that said treated callus was 6.83 while those at 30^{o}C were less than half (2.75). However, the relative percentage of both treatments used for anticipating efficiency of treatment was calculated as 100 and 40.29 for 25^{o}C and 30^{o}C grown culture, respectively. Figure 16 illustrated the friable desired callus grown at 25$\pm$2^{o}C (Table 11).

Callus maintenance

In an earlier study it was demonstrated that leaf-derived callus which had been established on MS basal medium containing 2,4-D and BA (1.0 : 3.0 mg/l) under 2,000 lux of fluorescent illumination about an 8-hr dark period and maintained at the temperature range of 25$\pm$2^{o}C was the most appropiate conditions for pyrethrum culture.

Therefore an examination on the growth and developmental stage in the growth pattern and morphological change of established callus

(A) (B)

Figure 16 Effect of temperature on callus initiation from leaf which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25 ± 2 ^{o}C (A) and 30 ^{o}C (B).

culture were carried out. The growth and developmental stage of callus could be designated as stage 1, 2 and 3 which respectively corresponded to yellowish green, yellow-brown and brown callus in colour, friable, semi-compact and compact-callus in friability (Figure 17).

Recording of time corresponding to the visual observation, color and friability, had also illustrated in Figure 18. To determine the growth pattern of callus culture it is necessary to obtain the GI value (fresh weight basis) and a morphological proporties which was obviously mentioned.

The data obtained after a culture period of 8 weeks were classified as follows:

First stage : Yellowish green callus with friability, culture time was 4 to 4 and half weeks onward starting from 0.

Second stage : Yellow-brown callus with less friability, culture time was during 5 to 6 weeks.

Third stage : Brown-callus with no friability, culture time was approximately over 6 weeks.

Callus maintenance was manipulated by frequent subculturing within 4 weeks after inoculation onto the fresh medium.

Establishment and development of suspension culture

Suspension cultures were normally initiated by transferring pieces of active growing callus to a liquid medium which was agitated

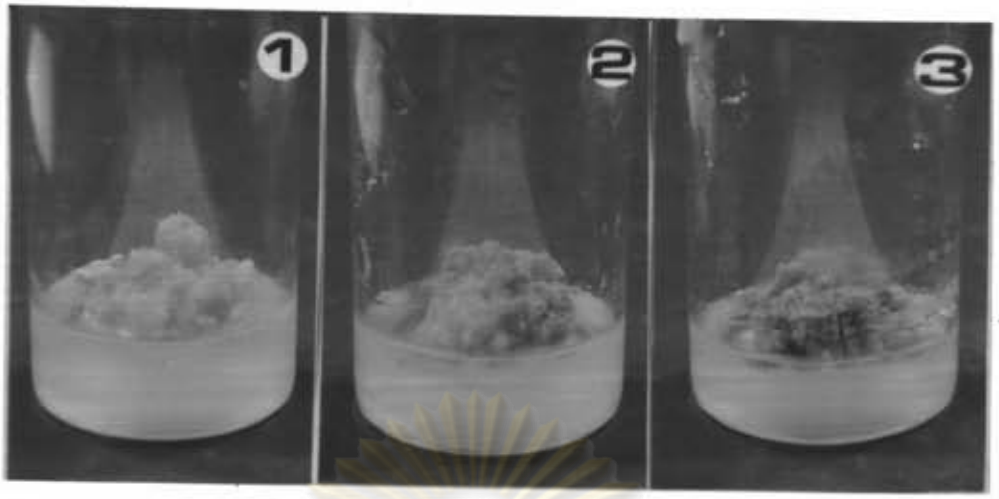


Figure 17 Growth and developmental stages of leaf-derived callus which had been cultured on MS basal medium containing 2,4-D (1.0 mg/l) and BA (3.0 mg/l) under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temparature range of 25 ± 2°C.
1 First developmental stage (yellowish green)
2 Second developmental stage (yellow-brown)
3 Third developmental stage (brown)

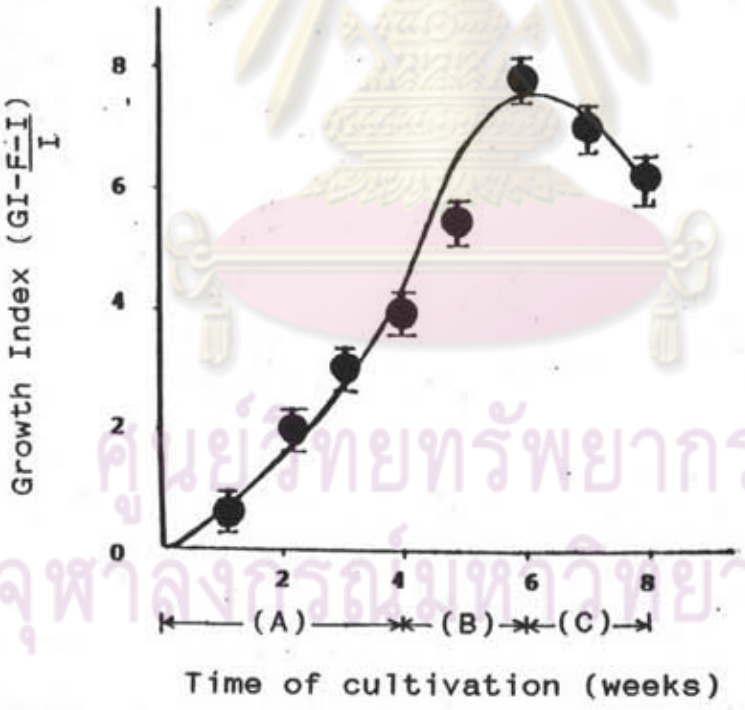


Figure 18 Growth pattern of leaf-derived callus cultured on MS basal medium containing 2,4-D (1.0 mg/l) and BA (3.0 mg/l) under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temparature range of 25 ± 2 °C.

(A)	First 4 weeks	First developmental stage
(B)	4 - 6 weeks	Second developmental stage
(C)	6 - 8 weeks	Third developmental stage

during incubation.

As previously mention, the case of establishment of suspension cultures from callus tissue was influenced by the friability of the callus tissue. Those following factors were examined for their effects on cell suspension establishment.

1. Free cell and aggregates of cells formation

1.1 Medium formulation

The culture medium used for suspension culture was usually based upon that which maintains good growth of callus, hence MS basal medium supplemented with 2,4-D and BA were chosen for establishing the cell culture. It also may, however, be necessary to modify this medium (particularly its growth regulator content good cell separation in liquid medium).

The differences due to the treatment of culture grown in MS medium supplemented with 2,4-D and with BA signified intensity of cell formation; treatment number C9 scored recorded the highest value (2.87) and cell formation score appeared to be 4.0 which was significantly superior to that of the control (0.0).

The obtained results could be categorized into four groups. First, C1 to C4 which were treatments grown in MS medium without 2,4-D supplement. This group it appeared to be no response of cell formation. Second, it seemed to be a little higher responsed than the first. Wide variation of GI value could also be observed from C6 and C7, C15 to C25 which comprised 0.21 - 0.95 GI unit and the scoring of cell formation value was in the range of 0.3 to 1.3. The third, the

highest value of GI units (2.25, 2.87) and 3.6, 4.0 units estimately calculated from score signifies intensity of cells formation were observed from 2 remaining treatments (C8 and C9). The last group gave less value of GI and cell formation score unit than the previous one, which were 1.29 to 1.79 and 1.3 and 2.3 respectively (Table 12).

Cultures grown in MS medium supplemented with IAA and BA (Table 13) comprised more wide variation of GI and score singifies intensity of cell formation value than the cultures grown in medium supplemented with 2,4-D and BA. Treatments number D13, D14 and D19 revealed the superior rank of those measuring units after 10 weeks of growth.

It is also evident from Table 13, the cultures grown in the medium without IAA supplement (D1 to D4) were found not to respond and the GI of remaining cell cultures were also not changed by the variation in concentration of growth regulator treatment and comprised little higher when compared with the cultures grown in 2,4-D and BA supplemented medium (Figure 19).

1.2 Environmental conditions

1.2.1 Light

In general, cell cultures require only low levels of light to regulate their morphogenic process. This study employed fluorescent illumination as the source of light to determine an effect of light on growth and the development of cell cultures.

Visual observation showed that extensive growth had occurred after culture period of 2 to 6 weeks and the culture

Table 12 Influence of growth regulators (2,4-D + BA) on growth and establishment of pyrethrum cell suspension culture

Treatment No.	2,4-D:BA	Score signifies * size of cell formation	Initial cell count δ (10^6 cells)	Final cell count δ (10^6 cells)	** GI
C1	0.00:0.0	0.0	4.00	3.7	0.00
C2	0.00:1.0	0.0	4.20	3.8	0.00
C3	0.00:2.0	0.0	4.14	3.7	0.00
C4	0.00:3.0	0.3	4.00	4.4	0.10
C5	0.00:4.0	0.3	4.18	4.3	0.02
C6	0.25:0.0	0.6	4.20	6.0	0.43
C7	0.25:1.0	1.0	4.10	7.5	0.83
C8	0.25:2.0	3.6	4.00	13.0	2.25
C9	0.25:3.0	4.0	4.00	15.5	2.87
C10	0.25:4.0	3.0	4.30	12.0	1.79
C11	0.50:0.0	2.3	4.50	10.2	1.26
C12	0.50:1.0	2.3	4.50	11.2	1.48
C13	0.50:2.0	2.3	4.40	10.2	1.32
C14	0.50:3.0	2.3	4.40	10.1	1.29
C15	0.50:4.0	2.3	4.10	8.0	0.95
C16	0.75:0.0	1.3	4.30	8.4	0.95
C17	0.75:1.0	1.0	4.00	7.0	0.75
C18	0.75:2.0	1.0	3.99	6.9	0.73
C19	0.75:3.0	1.0	4.00	7.2	0.80
C20	0.75:4.0	1.0	4.10	7.5	0.83
C21	1.00:0.0	0.6	4.20	6.8	0.62
C22	1.00:1.0	0.6	4.30	6.6	0.53
C23	1.00:2.0	0.6	4.20	6.0	0.43
C24	1.00:3.0	0.3	4.20	5.1	0.21
C25	1.00:4.0	0.3	4.00	5.0	0.25

0.5 g Leaf-derived callus were cultured in 25 ml MS liquid medium containing various concentration of growth regulators as previously illustrated under 2,000 lux of continuous fluorescent illumination and maintained at a temperature range of 25 ± 2 °C on a rotary shaker (100 rpm)

* 3 replications

** Growth index = $\frac{F - I}{I}$

Table 13 Influence of growth regulators (IAA + BA) on growth and establishment of pyrethrum cell suspension culture

Treatment No.	IAA:BA	Score signifies size of cell formation *	Initial cell count 6 (10 cells)	Final cell count 6 (10 cells)	GI **
D1	0.0:0.0	0.0	4.82	4.70	0.00
D2	0.0:1.0	0.0	4.35	4.20	0.01
D3	0.0:2.0	0.3	4.14	4.70	0.03
D4	0.0:3.0	0.0	4.01	4.38	0.00
D5	0.0:4.0	4.0	4.88	16.90	2.46
D6	1.0:0.0	4.0	4.80	23.90	4.00
D7	1.0:1.0	3.0	3.64	10.00	1.18
D8	1.0:2.0	0.3	4.35	7.18	0.65
D9	1.0:3.0	3.0	4.81	10.80	1.18
D10	1.0:4.0	3.0	4.37	10.60	1.44
D11	2.0:0.0	4.0	4.82	31.58	5.58
D12	2.0:1.0	4.0	3.65	15.60	3.33
D13	2.0:2.0	4.0	4.03	27.50	5.82
D14	2.0:3.0	4.0	4.24	31.10	6.32
D15	2.0:4.0	3.6	4.54	13.40	1.95
D16	3.0:0.0	0.0	4.22	3.87	0.00
D17	3.0:1.0	4.0	4.52	16.43	2.64
D18	3.0:2.0	4.0	4.67	17.34	2.72
D19	3.0:3.0	4.0	4.15	27.75	5.70
D20	3.0:4.0	4.0	3.93	16.56	3.22
D21	4.0:0.0	0.0	4.02	3.48	0.00
D22	4.0:1.0	0.0	4.48	3.89	0.00
D23	4.0:2.0	4.0	4.35	13.70	2.16
D24	4.0:3.0	0.0	4.00	3.27	0.00
D25	4.0:4.0	3.6	4.13	12.30	1.99

0.5 g Leaf-derived callus were cultured in 25 ml MS liquid medium containing various concentration of growth regulators as previously illustrated under 2,000 lux of continuous fluorescent illumination and maintained at a temperature range of 25 ± 2 °C on a rotary shaker (100 rpm)

* 3 replications

** Growth index = $\frac{F - I}{I}$

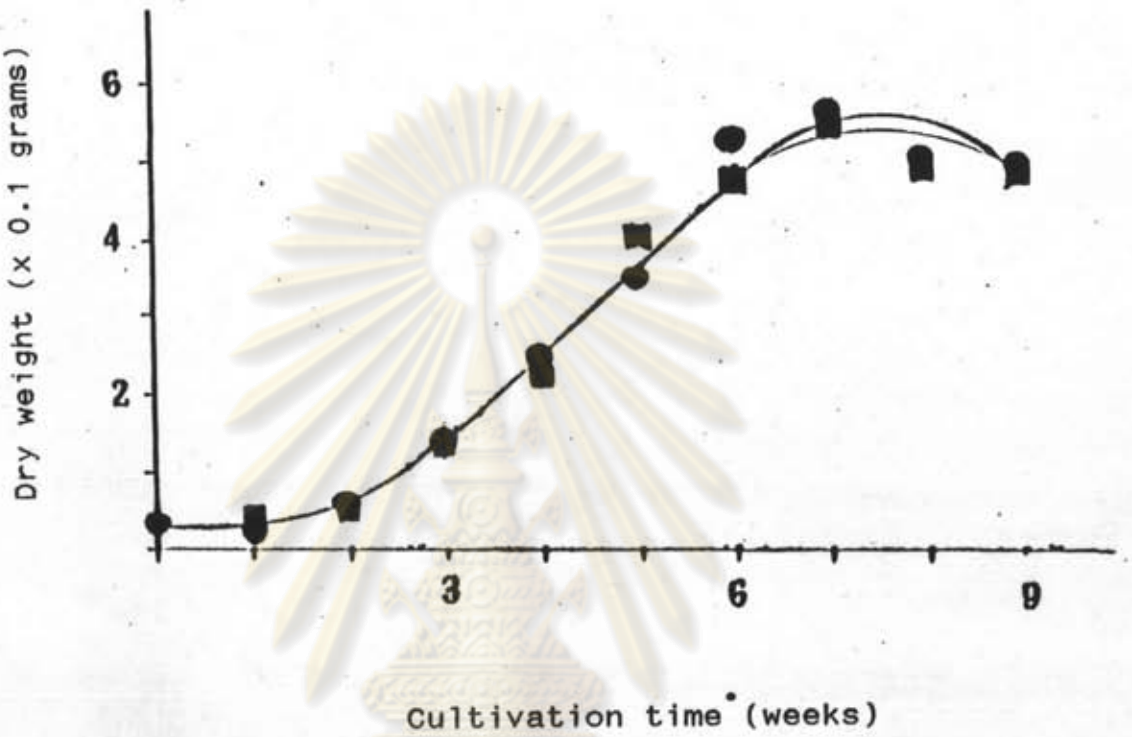


Figure 19 Growth pattern of 2,4-D:BA-grown culture (0.25:3.0 mg/l) (■) and IAA:BA-grown culture (2.0:3.0 mg/l) (●) which had been culture in liquid MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temparature range of 25 ± 2 °C.

density determined by a measurement of settled tissue fresh weight of Dark Grown culture comprised less than half of the another (1.38 and 2.83). Consequently, the relative percentage to GI was also comprised the same (48.57 and 100).

The differences due to treatment were also significant for the coefficient of variation, mean and standard deviation of 3 replicate analyses (Table 14).

1.2.2 Temperature

Data obtained from Table 15 exhibited that 25°C grown cultures gave significantly high GI unit (fresh weight basis) when compared with 30°C grown cultures. Mean and standard deviation of 3 replicate analyses of those GI units were re-evalutated and the result concerning the highest relative to GI percentage of 25°C grown culture was 100-fold of 30°C grown culture which had neglibly response. For the visual observation, the exhibition of brown-semicompact cell aggregates at the botton of culture fask and also change of culture medium to brown were recorded in cultures maintained at 30°C.

2. Batch culture on plateform shaker

The visual observation and determination of culture density by direct cell counting was study on growth and developmental stage of the established cells.

As is evident from Figure 20 that the adaptation of cells in the new medium was slow at the first and lag phasea and after 4 weeks. From this illustration, it was noticed that lag and early log phase which was designed to 1st stage of cell development was very long (2 to

Table 14 Influence of light and dark on the establishment of pyrethrum suspension cells that had been maintained under under different conditions*

a) Light Grown Culture

No. of replicate	Time of cultivation (weeks)						** GI	Relative to GI
	0	2	4	6	8	10		
1	4.00	5.50	6.75	16.25	16.25	15.50	2.87	
2	4.18	6.20	6.50	15.80	16.00	15.80	2.80	
3	4.30	5.70	5.70	17.30	16.30	16.50	2.83	
X	4.16	5.80	6.32	16.45	16.18	15.93	2.83	100
SD	0.123	0.294	0.448	0.628	0.131	0.419	0.029	

b) Dark Grown Culture

No. of replicate	Time of cultivation (weeks)						** GI	Relative to GI
	0	2	4	6	8	10		
1	4.00	5.20	6.90	10.30	9.70	9.44	1.36	
2	4.20	5.80	7.20	12.10	12.10	10.16	1,42	
3	4.00	4.95	6.50	10.98	11.20	9.40	1.35	
X	4.07	5.32	6.87	11.13	11.00	9.67	1.38	48.57
SD	0.094	0.357	0.287	0.742	0.990	0.349	0.031	

* Growth conditions as given in the materials and methods.

** Growth index (obtained from fresh weight basis) = $\frac{F - I}{I}$

Table 15 Influence of temperature on the establishment of pyrethrum suspension cells that had been maintained under different conditions*

a) 25 °C Grown Culture

No. of replicate	Initial fresh weight (g)	Final fresh weight (g)	** GI	Relative to GI
1	5.68	42.37	6.46	104
2	5.43	38.17	6.03	97
3	5.29	37.50	6.09	98
X	5.46	39.35	6.19	100
SD	0.197	2.64	0.24	

b) 30 °C Grown Culture

No. of replicate	Initial fresh weight (g)	Final fresh weight (g)	** GI	Relative to GI
1	5.30	12.82	1.42	96
2	5.65	13.50	1.39	95
3	5.27	13.72	1.60	109
X	5.40	13.34	1.47	23.75
SD	0.189	0.46	0.11	

* Growth conditions as given in the materials and methods.

** Growth index (obtained from fresh weight basis) = $\frac{F - I}{I}$

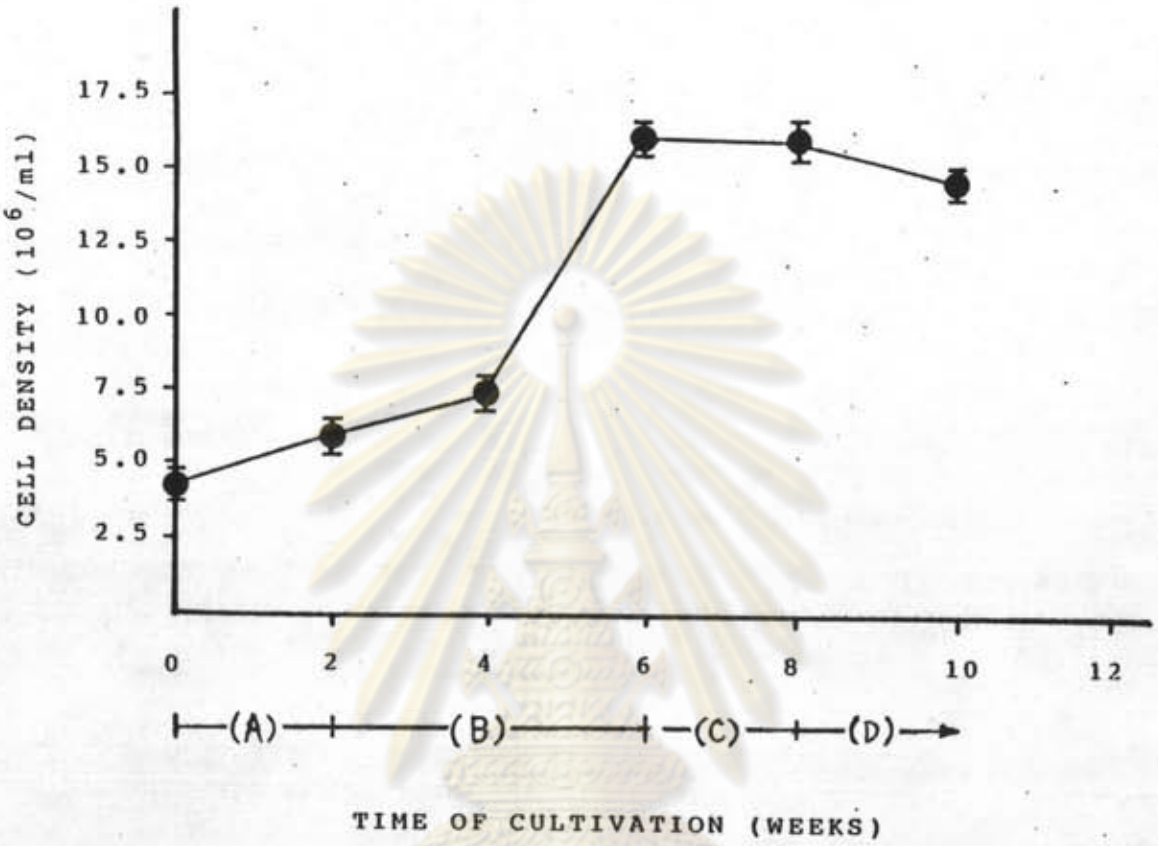


Figure 20 Growth pattern of suspension cells cultured on MS basal medium containing 2,4-D (0.25 mg/l) and BA (3.0 mg/l) under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temparature range of 25 ± 2 °C.

(A)	first 2 weeks	First developmental stage
(B)	2 - 6 weeks	Second developmental stage
(C)	6 - 8 weeks	Third developmental stage
(D)	over 8 weeks	Fourth developmental stage

4 weeks). The maximum of cell density ontained through a directly count, reached a final cell density of ca. 15 x 10^6 cell/ml 4 weeks onward. Logarithmic stage of cells had been noticed as the stage of the most active culture cell and their accerelation revealed rather low, that is, it had to take weeks to reach a num,ber of for raising up 2.5 to 15 x 10^6 cells. Not more than four successive doubling to the initial population had been conclusively recorded at the final results.

Suspension maintenance

According to the previous study, 3 stages of cell development was defined, base on their own characteristics.

First developmental stage : Culture age was first 2 weeks, visual observation was unclear-yellow cell suspension culture and abundant cells free occurred

Second developmental stage : Culture age was during 2 to 4 weeks

Third delvelopmental stage : A little change of the color to brown (6 to 8 weeks) and small cells aggregates could be found by visual observation.

Fourth developmental stage : Growth rate was declined when starting over 8 weeks of cultivation time, color is absolutely changed to brown and large cells aggregates appeared at the bottom of the culture flasks.

For suspension maintenance, the culture subculturing levels had been studied and is event that 4 to 6 week subculturing time was noteworthy because during the declining stage occuring of the cytoplasm leakage appeared abundantly (Figure 21).

Extraction of pyrethrins

1. Selection of extracting solvent

Conventional methods for extracting pyrethrins employed the extracting with three volatile organic solvents (petroleum ether, chloroform, and acetone) by shaking for at least 7 hrs in the Erlenmeyer flask on the mechanical shaker. Only petroleum ether extract yielded a transparent and solvent free extract which could be concentrated in one step. Further, each fraction was dried separately in a vacuum oven at 60^{o}C and the oleoresin was reconstitued in 1,000 ul with acetone before subjected to GC column. The results of an experiment were demonstrated in table 16. The obtained data exhibited that petroleum ether extract gave the first rank in yielding pyrethrins quantity, followed by chloroform's and the remaining acetone's was found to yield a product containing the lowest.

2. Selection of extracting procedure

Attempt to improve the extracing methods for yielding the highest pyrethrin quantity had been considered by many researchers since 1930. This study designed four conventional procedures as given in Materials and methods for extracting pyrethrum flowers.

Figure 21 Growth and development of 4 weeks-old pyrethrum suspension culture

A Log phase-suspension cell which had been cultured on MS basal medium under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25 ± 2 °C

B First and second stage cells

C 8 weeks-old suspension cell.

D Third stage of suspension cell occuring of some cytoplasm leakgage.

Table 16 Apparent scores of pyrethrin I extracted* by selection treatment

Extracting solvent			Pyrethrin I/Methylstearate (peak area)**		
Categories	Group	Polarity	Arithmetic mean value	SD	Coefficient of variation
1. Petroleum ether	1	2.9	0.6384	0.0036	0.57
2. Chloroform	9	4.3	0.6589	0.0338	5.13
3. Acetone	6	5.4	0.6294	0.0010	0.19

* Extracting method as given in the materials and methods
** Mean and standard deviation of 3 replicate analyses

Table 17 Apparent scores of pyrethrin I extracted* by selection methods

Extracting method	Pyrethrin I/Methylstearate (peak area)**		
	Arithmetic mean value	SD	Coefficient of variation
4 °C/3 days	0.6347	0.0069	1.09
25 °C/3 days	0.6337	0.0050	0.78
40 °C/3 days	0.6233	0.0093	1.51
Soxhlet 65 °C/7 hrs	0.6384	0.0036	0.57

* Use of petroleum ether as extracting solvent
** Mean and standard deviation of 3 replicate analyses

The conclusive results in Table 17 revealed that pyrethrum flower extracted with petroleum ether in Soxhlet apparatus yielded the product containing the highest quantity, followed by immersing in a Erlenmeyer flask at the temperature of $4^{o}C$. Mean and standard deviation of 3 replicates analyses of the remaining two, exhibited that extracting with petroleum ether at the lower temperature of $25^{o}C$ got higher yield than another (40^{o})

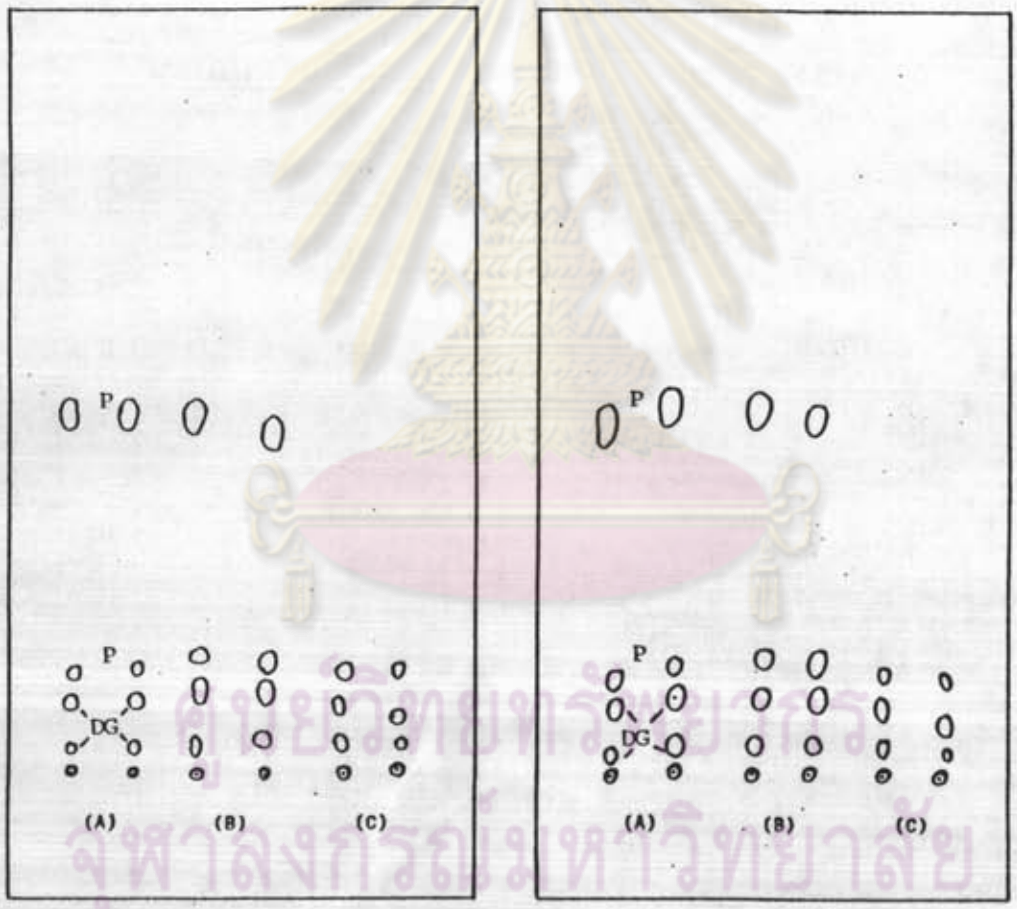
Qualitative analysis of pyrethrins by TLC

A refined pyrethrum extracts were prepared by taking about 5.0 g. dry wt of super-fine pyrethrum flower and callus powder as indicated in Figure 9 and the resultant residue was then redissolved in acetone before subjected onto TLC plates which were developed by 8 developing solvent systems. The conclusive results were illustrated in Figures 22 to 25, developing solvent system of n-hexane-n-heptane-EtoAc (48:40:12) gained the best resolution and high response to spray reagent, p-anisaldehyde sulfuric acid, followed by solvent system 10 % EtOAc in petroleum ether. From the fore gosing, the compounds appeared similarly to the references, pyrethrin I and II gave dark gray and dark gray-brown with Rf value 0.518 and 0.169 respectively when plate was developed under the previously mentioned developing solvent system. Whereas, pyrethrin I and II gave Rf 0.50, 0.20 (Figure 23) under developing system 75 % benzene in petroleum ether corresponding to respective standard compound. The limit of detection of this technique was 50 ng of each.

Figure 22 Appearance of TLC Chromatography of PBK Standard Solution, World Standard Solution and Crude Shirayuki I Callus Extract which was developed within these conditions.

a) Absorbant : Silica HF 254+336 Art 7739
Solvent system 70% benzene in petroleum ether (60-80°C)
Spray Reagent : p-anisaldehyde-sulfuric acid

b) Absorbant : Silica HF 254+336 Art 7739
Solvent system 75% benzene in petroleum ether (60-80°C)
Spray Reagent : p-anisaldehyde-sulfuric acid



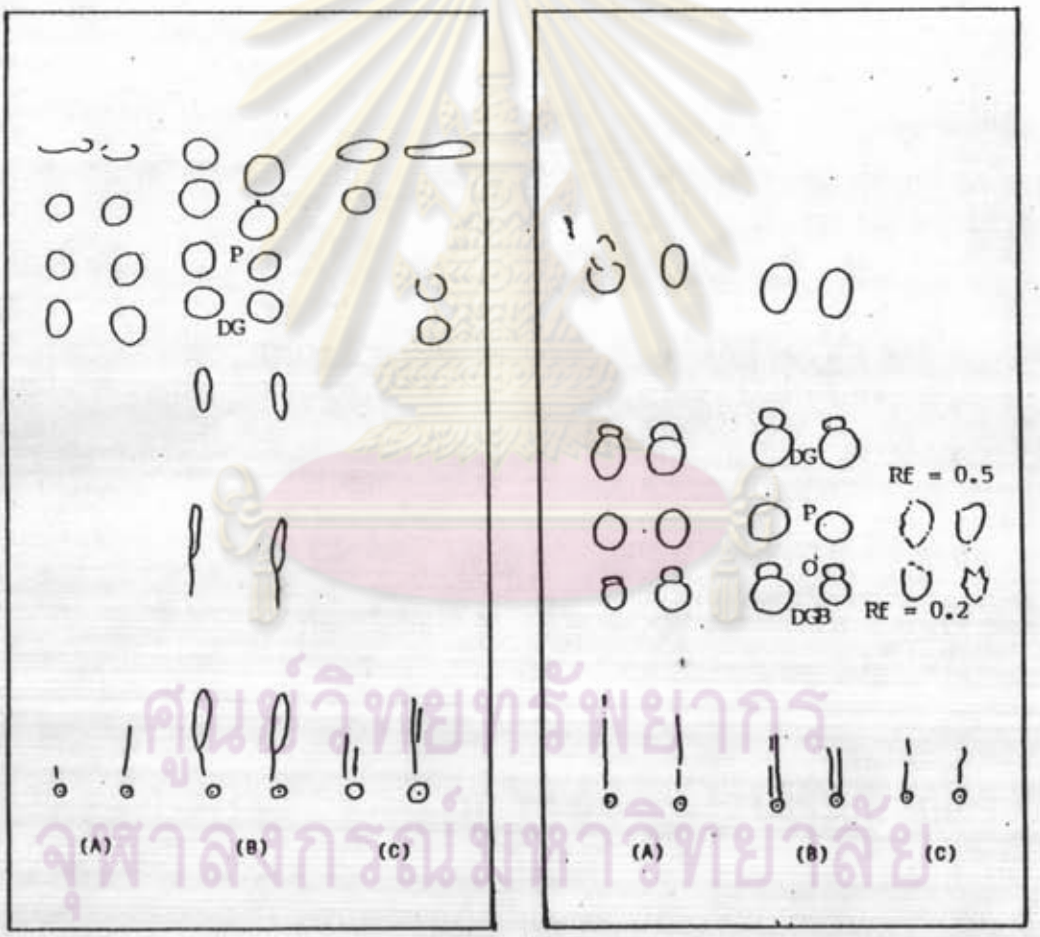
P = Purple
DG = Dark grey

(A) PBK (Standard)
(B) Word pyrethrum extract (Standard)
(C) Shirayuki I (Callus)

Figure 23 Appearance of TLC Chromatography of PBK Standard Solution, World Standard Solution and Crude Shirayuki I Callus Extract which was developed within these conditions.

a) Absorbant : Silica HF 254+336 Art 7739
Solvent system 4:1 petroleum ether (30-60°C)-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid

b) Absorbant : Silica HF 254+336 Art 7739
Solvent system 9:1 petroleum ether (30-60°C)-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid



P = Purple
DG = Dark grey
DGB = Dark grey brown

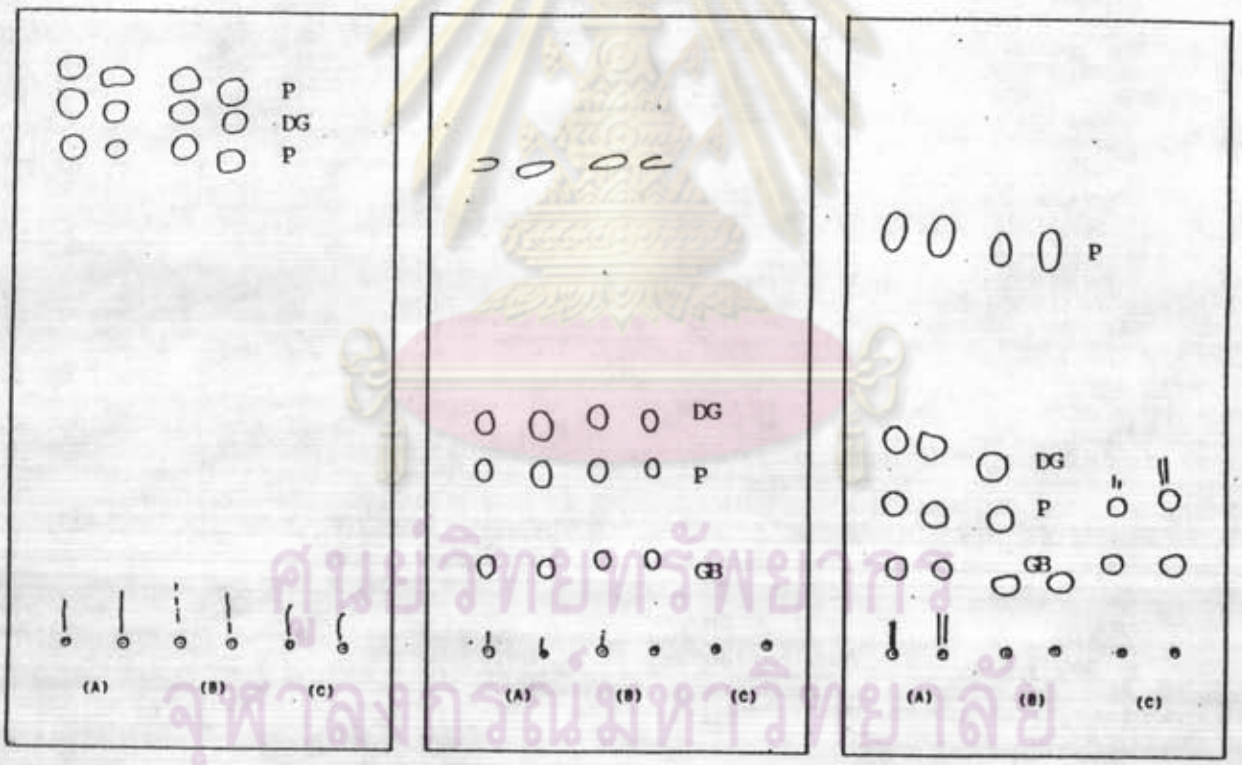
(A) PBK (Standard)
(B) Word pyrethrum extract (Standard)
(C) Shirayuki I (Callus)

Figure 24 Appearance of TLC Chromatography of PBK Standard Solution, World Standard Solution and Crude Shirayuki I Callus Extract which was developed within these conditions.

a) Absorbant : Silica HF 254+336 Art 7739
Solvent system 75/25 n-hexane-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid

b) Absorbant : Silica HF 254+336 Art 7739
Solvent system 85/15 n-hexane-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid

c) Absorbant : Silica HF 254+336 Art 7739
Solvent system 90/10 n-hexane-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid



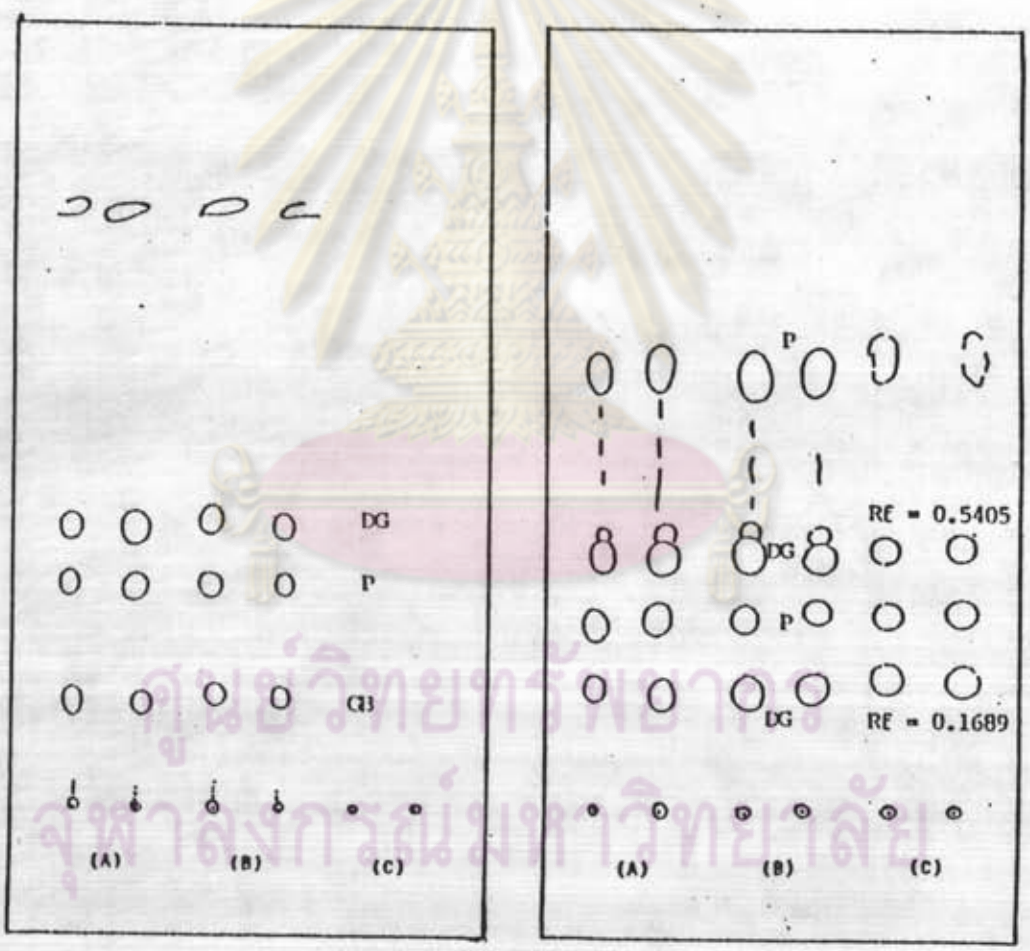
P = Purple
DG = Dark grey
GB = Grey brown

(A) PBK (Standard)
(B) Word pyrethrum extract (Standard)
(C) Shirayuki I (Callus)

Figure 25 Appearance of TLC Chromatography of PBK Standard Solution, World Standard Solution and Crude Shirayuki I Callus Extract which was developed within these conditions.

a) Absorbant : Silica HF 254+336 Art 7739
Solvent system 65/15 n-hexane-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid

b) Absorbant : Silica HF 254+336 Art 7739
Solvent system 48:40:12 n-hexane-n-heptane-ethylacetate
Spray Reagent : p-anisaldehyde-sulfuric acid



P = Purple
DG = Dark grey
GB = Grey brown

(A) PBK (Standard)
(B) Word pyrethrum extract (Standard)
(C) Shirayuki I (Callus)

Quantitative of pyrethrin by GC.

The separation of the "Pyrethrins" by gas chromatography required at least two important factors affecting the performance efficiency; the column temperature and the carrier gas (N_2) flow rate. The results of GC determinations which were carried out with temperature range of 150 to 225^{o}C was shown in Table 18.

The first component was eluted within six minutes and pyrethrin I and II appeared at 17.6 and 72.6 min. when performed at the lowest temperature of 205^{o}C. Conversely, if the temperature was raised at intervals to 225^{o}C to drive fast, cinerin I and jasmolin I were disappeared from the chromatogram.

In case of the flow rate, the isothermal plot of obseved HETP in relation to flow rate for carrier gas (N_2) yielded a characteristic hyperbola with a minimum HETP (Table 19 and Figure 26). In addition, under the experimental condition specified in this report, the chromatographic profile of six components was shown in Figure 27 and the pyrethrin I and II component of the insecticide fraction was the predominant peak recorded by the gas chromatograph.

The amount of pyrethrins contents in the callus extract was correspondingly determined by using the standard calibrating curve (Figure 28). Illustration number 29 indicated that the pyrethrum callus extract containing pyrethrins could be verified by gas chromatography.

Table 18 Retention time of pyrethrin I and pyrethrin in relation to column temperature.

Column Temperature (^{o}C)	Retention Time	
	Pyrethrin I	Pyrethrin II
150	-	-
170	-	-
190	-	-
205	17.6	72.4
215	16.0	70.2
225	7.6	30.4

Table 19 Apperance seores of retention time of methylstearate in relation to flow rate of GC operation.

Flowrate ml/min.	Peak width$^{1/2}$	Retention time(min)	*N=5.54($tr^2/w^{1/2}$)	@ HETP=L/N
28	12.0	247	2347.2	0.8521
29	10.0	210	2443.1	0.8186
30	9.0	195	2600.7	0.7690
31	9.0	177	2142.7	0.9334
32	9.0	174.5	2082.6	0.9603
33	9.0	165.0	1862.1	1.0741
34	8.0	149.0	1921.7	1.0407

Note : *N = Theoritical plate

@ HETP = (Length of column, cm.)/(retention of air, sec.)

Table 20 HPLC analysis of pyrethrins content in suspension cells in relation to GC analysis.

Chromatographic technique	Cultivation condition	Py I content (mg/g.dry wt.)	% relative
HPLC (C18, Merck)	2,4-D, BA (0.25/3.0)	706.07	100
GC (3% OV-17)	2,4-D, BA (0.25/3.0)	355.76	50.38

N.B* - calculated by computer via video chromatography control center model PU4895 LC interface.

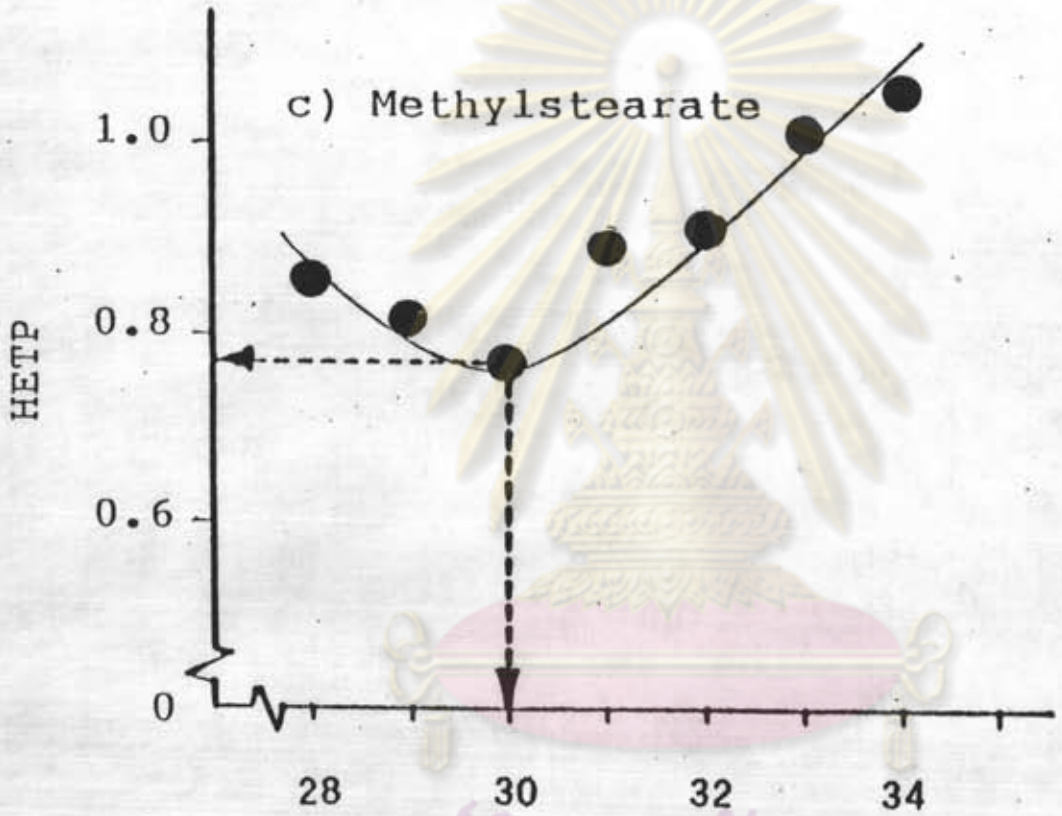


Figure 26 An isothermal plot of HETP in relation to flow rate of carrier gas (N_2)

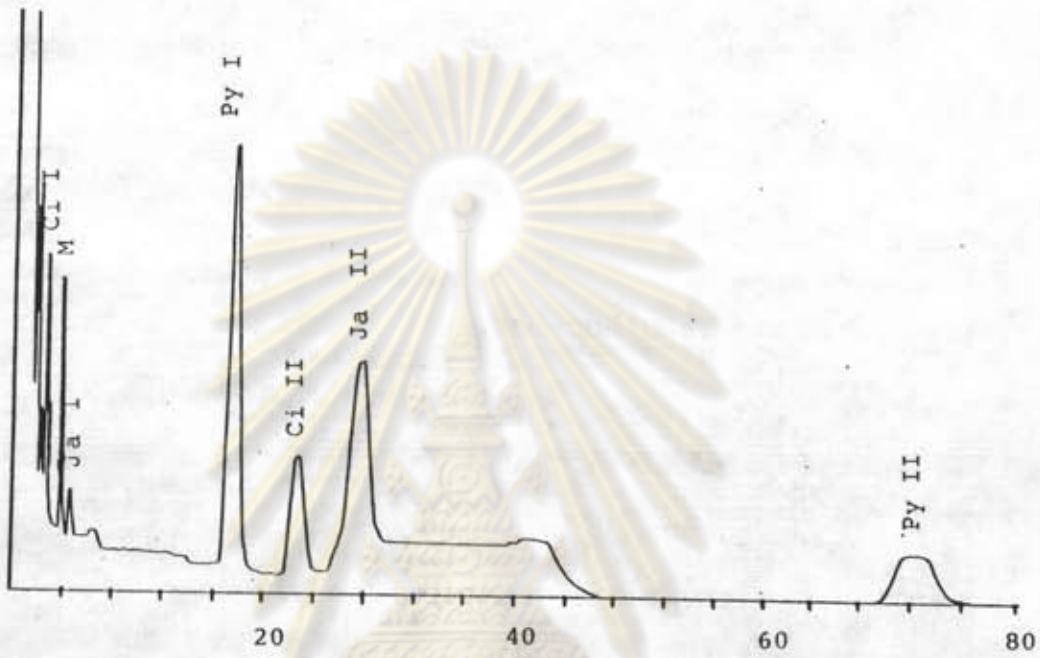


Figure 27 The GC separation of a mixture of the six pyrethrins components; equipped with flame ionization detector and 2,000 cm x 3.175 mm i.d. stainless steel column packed with 3% OV-17 on 80-100 mesh Chromosorb W(HP) (Varan 3700, Varian Associates).

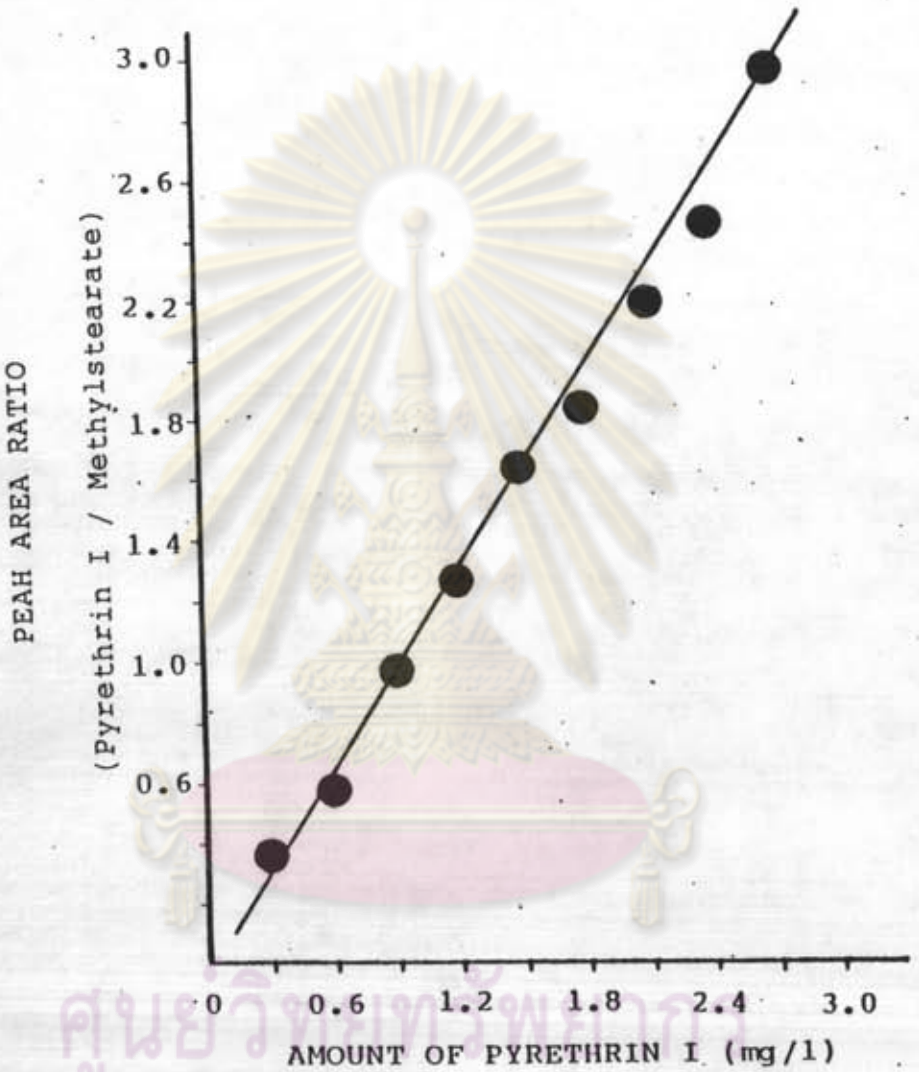


Figure 28 GC standard calibrating curve of word standard pyrethrum extract.

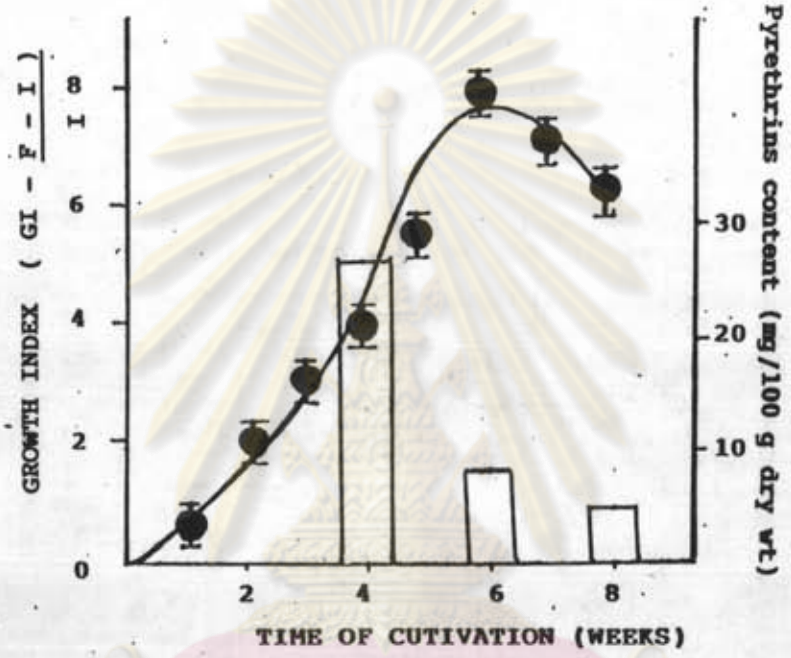


Figure 29 Growth and pyrethrins quantity of leaf-derived callus cultured on MS basal containing 2,4-D (1.0 mg/l) and BA (3.0 mg/l) under 2,000 lux of fluorescent illumination about an 8-hour dark period and maintained at temperature range of 25±2°C in relation of age of culture.

1. First developmental stage (yellowish green)
2. Second developmental stage (yellow-brown)
3. Third developmental stage (brown)

Quantitative analysis of pyrethrins by HPLC

According to the result of GC analysis described in Figure 27 is time-consuming and could not precisely determined amount of pyrethrins contents in culture cells if the available sample was lower than 5.0 g dry wt.* This aspect could be solved by useing HPLC. The use of six mixture of solvent system as eluting system revealed that MeOH:H_2O (1:3) and the flow rate of 1.0 ml/min comprised the best separation of all that insecticide.

In general, the production of secondary metabolites in plants is a complex process highly coordinated in space and time. Its main components are *biosynthesis* and *accumulation*which are usaully modified by tissue-and cell-specific compartmentation. Pyrethrins has been identified as 2^{o}metabolite containing in pyrethrum tissue. Figure 30 presented the chromatograms which two suspension cells extract which were appeared similarily in pattern (B and C). But for the natural flower extract, any impurities was not appeared at near the front of solvent (A).

Figure 31, illustrated the rate of growth and pyrethrins production of suspension cells which were cultured in medium containing 2,4-D:BA and IAA:BA at the concentration of 0.25:3.0 and 2.0:3.0 mg/ml respectively. The different pyrethrins contents were determined by HPLC analysis. It was evident that suspension cell cultured in medium containing IAA:BA (2.0:3.mgml) gave the high pyrethrins content in cells more than another.

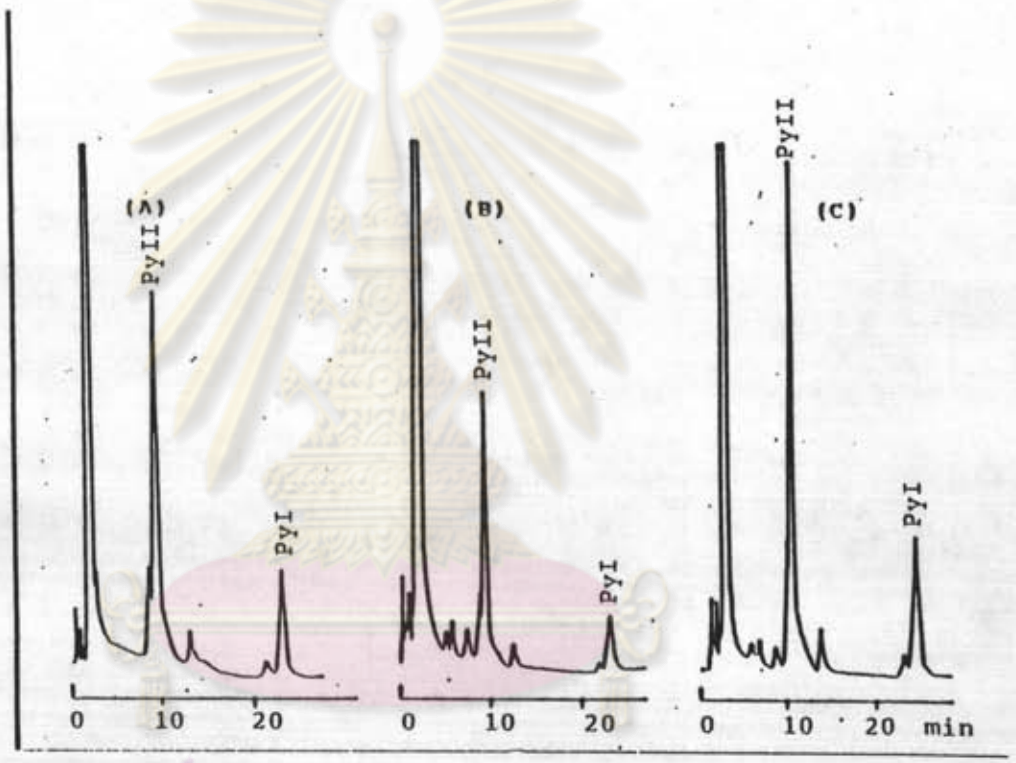


Figure 30 HPLC chromatograms of A, World standard pyrethrins extract, B and C represent pyrethrins extracts of suspension cells which had been cultured in MS medium supplemented with 2,4-D and BA (0.25:3.0 mg/ml), IAA and BA (1.0:3.0 mg/ml).

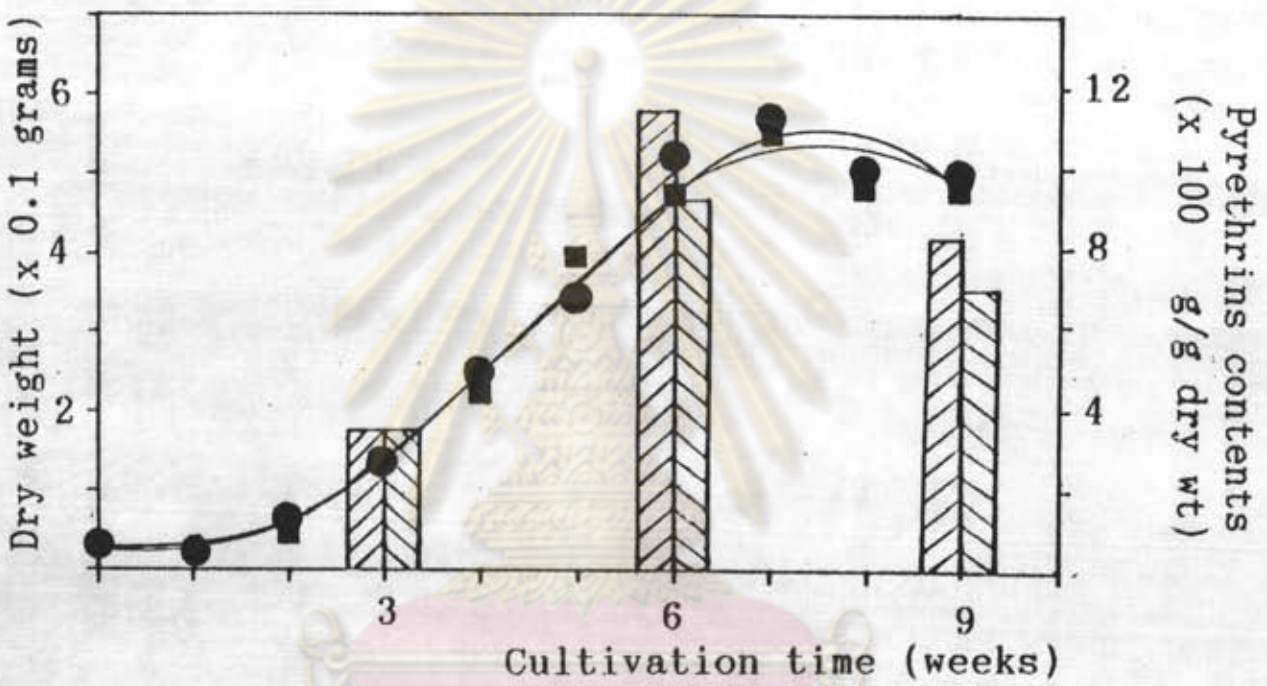


Figure 31 Growth and pyrethrins quantity in relation to the cultivation time of 2,4-D:BA-Grown culture (0.25:3.0 mg/ml) and IAA:BA-Grown culture (2.0:3.0 mg/ml)

● ■ Growth of 2,4-D:BA and IAA:BA

▨ ▨ Quantity of pyrethrins 2,4-D:BA and IAA:BA

As first above mentioned, the problem of unsufficient callus cells specimens created the unidentified pyrethrin content in cells by GC. Thus, the correlation of GC and HPLC analytical method were calculated for the ease of determination (Table 20). The data obtained the relative percentage for GC to HPLC was 50.38 : 100.

The change in ratio of pyrethrins constituents and stability of pyrethrins production in culture cells were finally examined. The results presented in Figure 32 to 33 revealed that one-year culture cells has some changes in ratio of constituent whereas the naturals still be the same. Although, the ratio of constituents were changed but the amount of pyrethrin I in one-year culture cells had been the same. Figure 34

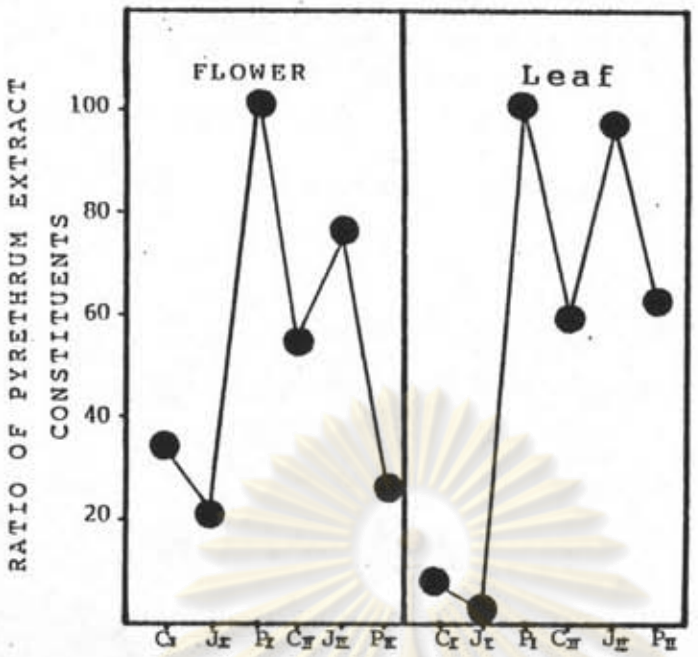


Figure 32 Ratio of pyrethrins contents extracted from natural dry flower.
C = Cinerins
P = Pyrethrins
J = Jasmolins

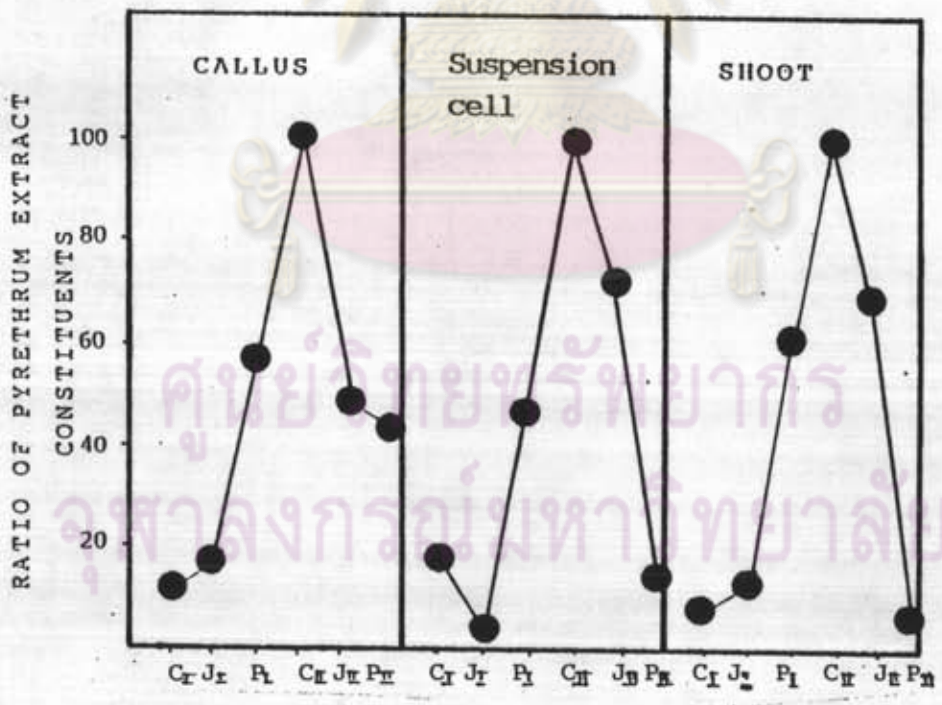


Figure 33 Ratio of pyrethrins contents extracted from three types of culture cells derived from leaf blade.
C = Cinerins
P = Pyrethrins
J = Jasmolins

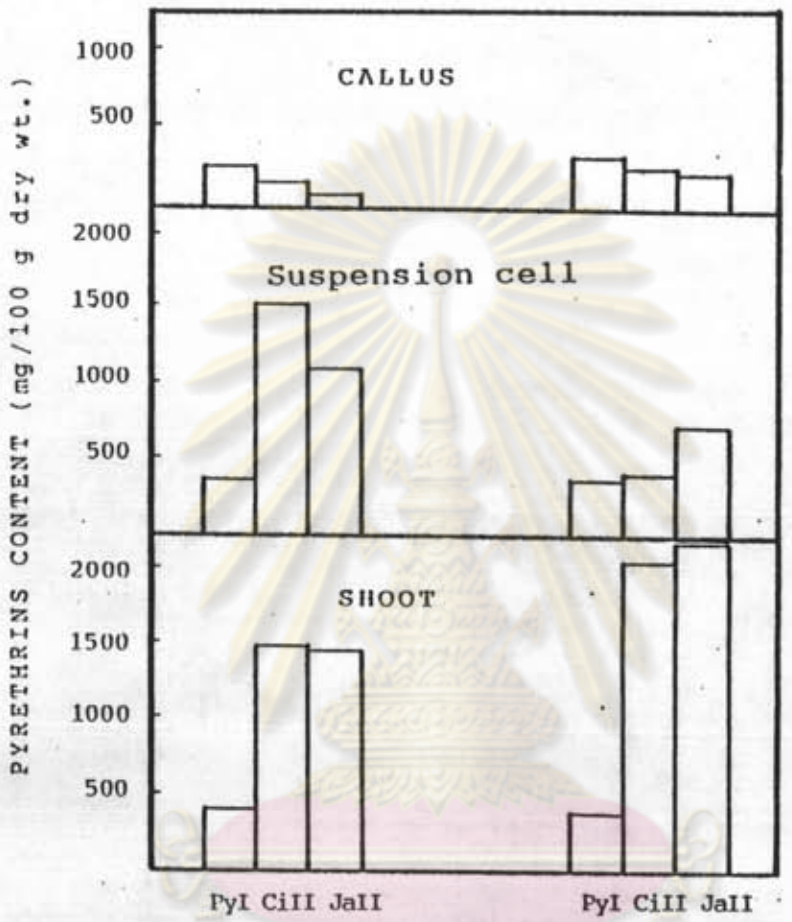


Figure 34 Stability of pyrethrins synthesized in one-year leaf-derived callus, suspension cell and shoot.
Py = Pyrethrin
Ci = Cinerin
Ja = Jasmolin