CHAPTER IV

DISCUSSION

Callus Initiation

1. Tissue sterilization

It has been observed from this study that surface sterilization is particularly difficult with hairy or unevenly surfaced materials as they harbor pockets of microorganisms. Occasionally a combination of more than one kind of disinfesting agent is effective. A compromise has to be achieved between adequate disinfestation and to cause as little damage as possible to the tissue cultured.

Pal (1985) reported that leaves and petioles collected from one-month-old seedlings which were placing in 20% NaOCl (v/v) for 5 min and washed five times provided satisfaction results. Kueh,*et al* also reported that placing the leaf laminae and petioles in 10% NaOCl and rinsed three times with sterile water gained sufficient surface sterilzation results (Kueh *et al*, 1985).

The data in Table 7 indicated that placing the tissues in 10% NaOCl (v/v) for 4 to 5 min and re-sterilized with 5% NaOCl (v/v) for 5 min before rinsed twice in sterile water removed all contaminants and caused no damage to cells,thus was fairly agreed to be the most efficient treatment. The differences between the highest levels of disinfestation and the lowest were statistically significant.

2. Establishing callus culture

2.1 Medium formulation.

Harrington and Smith (1977) suggested that the mechanism of uptake of a nutrient by plant cells can be anticipated to have some important consequences for growth rate and yield of cells. The mechanism may be considered as two cases. First, is the uptake by diffusion, and the second is an active uptake which is energy dependent or against a concentration gradient. For an active uptake, one can anticipate that the growth rate will be independent of concentration at relatively high external concentrations, that the final yield will be dependent on the amount of nutrients supplied, and that very little of the nutrient will remain in the medium after growth (Maretzki 1970, 1972 Smith 1976).

Growth regulators. There are several classes of compounds and several individual compounds which are known to have different regulatory effects on growth and development in the whole plants. These are auxins, cytokinin, giberellins, abscisic acids, and ethylene. Of these compounds or classes of compounds, the auxins and cytokinins are clearly those of the greatest significance in plant tissue culture.

And that, the greatest difference between tissue from different sources in terms of their behavior in tissue lies in the levels of auxin and/or cytokinins which are required for growth. This aspect of medium composition is the first to be examined in the initiaton of cultures.

According to the study of Skoog and Miller (1957), varying the concentration of these two GR in the culture medium of tabacco pith or callus tissue could acheive either unorganized growth, growth plus shoot formation, or growth plus root formation.

Choi *et al* (1981) reported that Ginseng(*Panex ginseng* C.A. Meyer) callus which was cultured on a medium containing 2,4-D and BA produced considerably higher amounts of saponins as compared to that produced on a medium containing others.

As an evidence in Figures 10 and 11 and Table 8, it was noticed that cultures grown on medium containing 2,4-D (0.5-1.5 mg/ml) yielded similar results to the previous report of Khanna (1976), Cashyap *et al* (1977), Jain (1977) and Zieg *et al*. In addition, four groups of explant response were classified and the fourth yielded satisfactory results, highest % Callusing (68-91%); ca. 3.0 scoring units and over 3.43 to 7.63 GI units. These might suggest that the most favorable concentration of 2,4-D for cultured callus of those leaf and petiole explants was 1.0 mg/ml.

In case of explants grown on medium containing no 2,4-D or unadequate concentration of 2,4-D , the callusing was absence. This may indicate that generally the role of auxins in cell division seems to be at some point in the G1 phase of the cell cycle because. In the absence of auxin, DNA synthesis is inhibited (Das *et al*, 1956; Nishi *et al*, 1977)

For the term, cytokinin, it is used to encompass compounds which promote cell devision in callus cultures of plant tissue. The compound has additional physiological effects in plants and

plant cells.

In test of the effects of 2,4-D and BA on primary explants, these two compounds are usually initially tested at the concentration of 0.01 to 10 mg/l and occasionally as high as 100 mg/l is noteworthy. According to the reports of Miller (1969), Torrey and Fosket (1970), when 2,4-D and BA are provided to tobacco or soybean cell cultures or pea root segments, systems which classically require both an auxin and cytokinin, the addition of exogenous cytokinin is not required.

In the opposite, for illustration number 12, it suggested the effects of 2,4-D and BA combination on pyrethrum leaf derived-callus Initiation that the addition of exogenous BA was not required (2,4-D and BA 1:0) but added cytokinin might be stimulatory. The highest concentration of BA (3.0 mg/ml) altered the rate of callusing from 26.6% to 91%. The increasing results had already been retested for three times. This may be discussed that cytokinin played a role in promoting the pyrethrum cell division (Figure 12).

Shabde and Murashige (1977) suggested that specific cells or tissue in plants produce auxins and cytokinins which are then transported to their sites of action to control growth and development, not all cells appeared to produce these compounds. That is, the majority of plant cell parts, when excised and placed in tissue culture, required an exogenous supply of auxin and/or cytokinin for growth and cell division.

Another point of view for the effect of 2,4-D and BA on callus initiation of each organ sources summarily illustrated in Figure 13. It appeared that leaf responsed to those regulatory factors in the highest level, followed by petiole and stem pieces respectively. This occurrence may be caused by whether the existing of GR itself in those tissues is sufficient for callusing response and/or the polarity of auxin movement. Usually, the polarity is markedly more evident in some tissues than in others, coleoptiles being usually very polar and some stems and petioles being less so. Thus, the ability of stems to transport auxin basipetally declines in a gradient down the stem and only small amount of auxin moved acropetally. In addition, it may be caused by the rate of the nutrient uptake through cells which some of them posess the outer cell wall coated by cutin and/or others protecting the transportation with caused more difficulty .

Salts requirement. Cases where the measurement of uptake of nutrients by plant cells in the culture has been made and the affinity of the transport system estimated are recorded (Appendix 10). For the usual nutrients of a medium, the affinities vary from 0.003 to 1500 uM. These transport systems will be operating at 95 % of their maximum velocity when the concentration of a compound transported is 19 times the affinity constant.

King and Olenick (1973) suggested that $NO_3^=$, L-alanine have, in common, very small affinity constants (46, and 225 uM , respectively) when the cells are starved and very large affinity constants when the cell have an excess of nutrient or an alternative. This might reflect scavenging mechanisms which can come into play when nutrients are depleted. Such scavenging would allow some continuation

of growth by the use of materials which were lost from the cells during the early part of growth. The assimilation of nitrogen can be dealt with in three parts of the reduction of NH_4^+ into organic compounds, and the interconversions of the nitrogen containing organic compounds.

For $SO_4^=$, it was reported by Reuveny and Filner (1977) that the first metabolic alteration in sulfate is the reaction with ATP in which pyrophosphate and adenosine-5'-phosphosulfate (APS) are the very important products (Reuveny and Filner, 1977).

Murashige and Skoog concluded that, despite a variability in tissue response to PO^{3-}_4 and an interaction with K^+ and perhaps also with sucrose and iron, 1.25 mM of PO^{3-}_4 was close to the optimal level for tabacco pith explants and callus culture. It seems, therefore, that the media formulated by Murashige and Skoog, was a dequately supplied to insure that carriers of that previous mention are operating at or near their maximum velocity.

GI obtained from cultures grown on 1/2 strength MS basal medium (Table 9) comprised quite low less than half of the another. It might be caused the fact that the by source of salts in medium was undequately supplied to enhance the carriers' operation in cells. For example, ATP sulfurylase, the enzyme catalyzing the reaction with ATP producing pyrophosphate and APS, is derepressed in tobacco cells under conditions of sulfur starvation or limited sulfur availability (Reuveny and Filner, 1977).

2.2 The enveronmental factors.

2.2.1 Light. From a splistic point of view, light is merely the form of radiant energy to which the human eye is sensitive. More precisely, it is a propagating, orthogonal electric and magnetic field (electromagnetic radiation) characterized by specific wavelength ( ) properties. The normal practice 25 years ago was to store sample tubes in the laboratory under prevailing light conditionss, periodic exposures to an illuminance of about 100 foot candle (fc.) Fortuitously, this level was adequate for sustaining growth, although at below the 350 fc optimal value (light source not reported) found by de Capite (1955) for callus growth in *Helianthus annuus, Parthenocissus tricuspidata* and *Daucus carota.* Jain (1977) reported that normal room light condition (periodic condition not declared) was also adequate for sustaining growth of pyrethrum callus culture. More recently as an interest in tissue culture research increased, particularly from the applications aspect, investigators began to recognize the basic role which light played in both the growth and development of the cultures.

Bickford and Dunn (1972) and Went (1957) stated that the characteristics of radiation which influence plant development in general are also those which affect plant tissue in culture. These aspects are generally classified as intensity, spectral quality, and length of the daily exposure period. Tissue cultures normally require red or blue light. Thus, light sources which emit light with this spectral regions will be effective in eliciting morphogenic responses. Plant are also sensitive to the number of hours of light to which they are exposed each day. So pyrethrum tissue cultures which was examined for effect of light (fluorescent illumination) may be effective in the

same way. Fluorescent source generate UV light (253.7 nm) in a long-arc tube by the excitation of low-pressure mercury gas. The UV light then excites a phosphor material which coated on the inner surface of the arc tube or tubular glass envelope. The phosphor subsequently emits specific wavelengths of fluorescent light (>253.7 nm), depending upon the phosphor. For the purpose of this study, then, is to briefly investigate that light does indeed influence the growth and development of pyrethrum callus cultures.

In the evidence of our data obtaining in Table 10 , rapid callus initiation, with two fold increased was observed to occur under the photoperiodic condition of an 8-hour dark period (referred to Solid light grown cultures) and callus proliferation noted by GI unit was meagre two-fold in darkness(referred to Solid Dark grown culture).

Seibert (1975) and Weis and Jaff (1969) stated that tobacco callus growth and shoot initiation can, in fact, be either enhanced or inhibited depending on the wavelength and irradiance. In growth effect, under normal tissue culture conditions, callus cultures are known to develop chloroplasts in the light, carry out photosynthesis (Neumann and Raafat, 1973) and evolve oxygen (Neumann *et al*, 1969). However, the presence of sucrose in the culture medium inhibits both chlorophyll synthesis and photosynthesis and photosynthetic carbon fixation in tissue cultures (Neumann *et al*, 1969). Thus, what photosynthesis does occur cannot support the growth and development in the callus culture. Therefore, the observation of our culture's characteristics, friable yellowish-green callus, may be due to the photomorphogenic rather than photosynthetic effects of light.

2.2.2 Temperature. Carew and Staba (1965) stated that studies on the *in vitro* growth of plant cells have indicated that the optimum temperature is generally within the range of 25 to 30°C, but the species could differ considerably. Matsumoto (1972), using cultures of *Populus* and *Nicotiana*, observed that growth was better at 32°C than at either 28 or 24°C. Rose and Martin (1975) made an extensive study of the growth of *Ipomoea* cells at temperature range of 15 to 34°C. Maximum growth occurred between 25 and 32°C, with temperature variations within this range having little effect on growth rates based on dry cell yeilds. On either side of this range, the growth rate declined dramatically.

It was noted from our data presented in Table 11 that growth rate of pyrethrum callus cultures grown under continuous dark condition provided less than half of one that grew under the photoperiodic condition. This occurrence may be discussed for either the lacking/declining of phenomena of salt transportation in cell or declining of temperature-dependent enzymatic metabolism. According to reports of Pal and Dhar (1984 and 1985), the effect of temperature on pyrethrum callus cultures were examined and the conclusive result had also revealed the maximum rate of growth and the pyrethrins production in cell were found in culture maintained at 25°C. Discussion of this results may be supported by the observation for the rate of sucrose and amino nitrogen utilization which was maximum between 30 to 32°C. Both of these parameters declined by about 25% from 30 to 25°C, whereas the growth rate declined very little. In addition, it was observed that the nitrogen utilization was reduced to a greater extent than was sucrose utilization at temperatures below 25°C. This indicates

temperature-dependent shifts in metabolism.

Callus Maintenance

As an evidence illustrated in Figure 19 and 20, the conclusive results of visual observation base on color and friability corresponding to time of cultivation revealed three stages of callus development. It was found to be certainly changed of their morphology within the limited time. The maximum growth rate was reached after 5 weeks and suddenly declined in the 6th week. Probably, callus was controlled under the culture treatment for too long, and that may be the result of diffusion being a rate-limiting process in those media.

With respect to subculturing level reported by many reserchers, according to Jain (1977), the tissues were subcultured in fresh medium every 6-8 weeks, whereas frequent subculturing for callus maintenance was also performed in Cashyap *et al* 's experiment.

In addition, during the course of growth of plant cells in media which are in general use, the pH of the medium changes ; thus, it may drop as low as pH 7 (Wetherell and Dougall, 1976; and Gamborg, 1970). Such changes can be expected to have an impact on the metabolism of the cells.

Establishment and Development of suspension culture.

The objective of this study is to investigate an available information on nutrition and environmental factors effecting the establishment and development of suspension culture inducing also secondary metabolite production.

1. Free cells and aggregrate cells formation

1.1 Media formulation

In accordance with the previons study, a successful establishment of the suspension culture depends on the initial callus being friable and it may be necessary to use an appropriate level of growth ragalatas in the callus culture medium to achieve the necessary friability of callus.

In recent years, suspension cultures are suitable for studying on the regulation of secondary metabolism in relation to grow, since cells cultured in this manner are relatively hormogenous and environmental conditions can be easily controlled. Moreover, it is possible to analyse the relationship between secondary metabolite production and growth in terms of cell number and cell morphology.

1.1.1 Organic Constituets

Growth uguletors, is one of the most important factors controlling growth in plant cell undergo not only the morphological changes during culture but also intracellutar metabolic changes.

Auxins may be put in ordered in turn of increasing activity as IAA, IBA, NAA, CPA, and 2,4-D, as a general summation of their efficiency. Investigation of cell suspension establishment of our present study, cetilixed of 2 kinds of auxin 2,4-D, a synthetic auxin, compared with IAA, a natural one for determination of those said GR influencing in cell culture. The summarixed results exhibited the limiting growth rates in both cultures were higher than culture obtained on solid media. This may due to rate of diffusion process in the liquid medium was absolutely higher.

In comparision of 2,4-D : BA and IAA:BA treatment, Figure 21 illustrated the growth pattern (base on fresh wt) that 2,4-D grown culture was a little bit lower than the IAA-Grown culture which may be replained that

After the 1st step of establishing free cells, the culture may form readily and does not required a period of adaptation when they were transferred to new medium (IAA and BA).

It was reported by many researcher e.g Furuya et al (1971), Tabata et al (1971) that synthetic auxins, especially 2,4-D, inhibits the production of nicotine synthesis in *Nicotiana tabacum* cultures. Zenk *et al* (1975) also state the inhibition of authraquinones production in *Morida citrifolia* cultures etc. These support the previous discussion.

1.2 The environmental condition

1.2.1 Light. The effect of light to the increasing of cell suspension density was still uncleared. Pearson (1978) reported on the increasing of fresh weight of Nicotiana tabacum cell suspension cultivated under a continuous dark had the effect on the nicotin production in cells.

Forest (1969), Davies (1972 and 1974) found that light had effect on Paul's Scarlet Rose and Tea plant cell suspension initiation. Corduan and Reinhard (1972) found that light and quality of light had an effect on cell culture and volatile oil producing induction in *Ruta graveolen*.

In this study, results obtained in Table 14 were also agreed with finding of several reserchers in this particular line (Kuch 1985 ; Staba , 1984 and 1985).

1.1.1 Temperature. The role of temperature in sustaining growth and metabolite production was still unclear. An attempt to investigate this problem, many reserchers try to examine how it effects to those phenomena.

Tulecke and Nickell (1960) examined the effect of temperature on five suspension cultures. The extreme optimum were 20 to 21^{o}C (Solium) and 31 to 32^{o}C (Rosa). Erikson (1965) found that Haplopapus gracilis cell suspension cultures grow several times faster at 30 that at 25^{o}C.

Pyrethum (Chrysanthemum cinerariaefolium) suspension culture were employed to observ growth and production of pyrethrins when it was maintained at 25^{o}C.

It was also evident that leaf-derived suspension culture maintained at 25^{o}C gave better growth than those of 30^{o}C (Table 15). This suggest that temperature over 25^{o}C, may either shift/stop enzyme activity and/or metabolism of cell.

2. Batch culture on plateform shaker

As evidence in Figures 21 and 22, batch-grown culture exhibited ideal sigmoidal growth in three phases, i.e., the lag, logarithmic (log,exponential) and stationary phases. In general, suspension cultures in the logarithmic phase consist of small, round cells or cell clusters which develop to form larger cell aggregates as a consequence of active cell devision, while most cells in cell clusters

are composed of expanded or elongated large cells. In contrast, high cell density of subculture brought about a limited number of cell division before entering the stationary phase (Figure 22).

Suspension Maintenance

Stages of cell development of leaf derived suspension culture of pyrethrum are illustrated in Figures 22 and 23, exhibition of various visual observations were also recorded.

During the course of growth of plant cells in media which are in general use, the pH of the medium changes may be expected to have an impact on metabolism of cells. Third stage (culture age over 8 weeks old) culture medium exhibited few large clumps of cells at the bottom of the culture flask. This may be results of either the change of pH and/or accumulation of unsufficient nutrient in media and/or pH change such above previously mentioned. Moreover, this may be supported by Figure 22 when cells undergo, not only morphological changes during culture but also intercellular metabolic change. In addition when cells entered to the late stationary phase, some cell leakage had also been observed.

Extraction of pyrethrins

1. Selection of extracting solvent

One of the earliest attempts to refine pyrethrum extract was described by Gnadinger and Corl (1932). Ground flowers were extracted with dichloroethane and the extract was heated to remove the solvent. The residue was dissolved in a suitable solvent, made up to the required

concentration of pyrethrins, and cooled to throw out resinous matter.

Preparation of purified pyrethrum concentrate from the whole pyrethrum flowers has been described by Jernakoff, Batt and Sankowsky (1952). Initial extraction of whole flowers with petroleum ether removed a relatively large amount of inert material containing only a small amount of pyrethrins. The flower residue was ground to a fine powder and extracted with petroleum ether. Removal of the solvent gave a refined concentrate containing about 45 % pyrethrins.

As was evident from Table 16 the extraction with petroleum ether, the solution existing in group 1, yielded the highest pyrethrins quantity. The results from this investigation indicated that pyrethrins itself is mono/di-carboxylicacid ester which posesses rather low polarity and among those solvents petroleum ether has been designed to be in group 1 with lowest polarity (2.9). In consequence, the suitable of partition reaction with petroleum ether should be higher when compared to the others.

2. Selection of extracting procedure

In general, mature flowers extract contained two fractions of extractives after it had been refined by solvent extracting. First fraction, 80% of the products, contained various fatty acids such as oleic, linoleic and palmitic; other vegetable extractive; and a small amount of petroleum distillated used to standardize the product. Another contained 20% insecticidal principle (Moore, 1979).

The comparision of purification processes, in term of Pyrethrins recoveries, must be made with care. Katsuda (1954) found

that during the extraction of pyrethrum flowers with petroleum ether, the higher, the temperature for extraction, the higher the concentration of resin in the extract.

Haus and Price (1962) used a purification process claimed to produce a biologically potentiated and non-staining pyrethrum extract. Crude pyrethrum extract was diluted to about 20% pyrethrins concentration with kerosene, extracted with 95% aqueous methanol and the temperature was lowered to 15°C to 20°C to facillilate the separation of the immiscible layers.

As the same aspects, our obtaining data in Table 17 suggested that the occurrence of low temperature extracting procedure provided a good agreement. This may be described as the ease of separation and participitation of some impurities of the first fraction.

Conversely, for consideration of extracting with petroleum ether in Soxhlet apparatus for at least 7 hrs, it was found to be the procedure yielding the product containing the highest pyrethrins quantity. This may be anticipated that the solvent removed by distillation yielded a concentrated extractive which may not be completely soluble in the petroleum distillate. To overcome this, the concentrated extractive was dissolved in lower aliphatic hydrocarbon solvent and then chilled to aid the separation of more impurities.

Qualitative analysis by TLC

Study on the active ingredients of pyrethrum extract and there constituents in callus. It was said that, initially the position or order of the respective "Pyrethrins" on the TLC plates could be

predicted on the basis of their structural composition. That is, the expected absorption of fluorescent affinity (Stahl, 1966). The initial assumption was that the higher Rf value, the fewer double bounds and the more-CH^2-groups a substance would possess. Jasmolins have one double bond less in the side chain of the five membered ring as compared with pyrethrins, while cinerins have one less-CH^2-group and this assumption had been reconfirmed by using GC.

As was evident from Fig 22 to 25 that results are fairly in agreement with those reported by (Stahl, 1966; Sandararajan and Chawla, 1983)

Quantitative analysis of pyrethrins by gas chromatography

As was evident in Table 18, 19, that appropriate conditions: column temperature (205^{o}) and 30 ml/min carrier gas flow rate which was a short analysis time at a low operating temperature in order to minimize thermal decomposition.

Therefore, the HETP in minimum rate (Figure 26) is the flow rate at which the column is operating most efficiently. The chromatographic profile described in Figure 27 shown good characteristics. Cinerin I was the compound which was eluted first, this may be caused of its lowest molecular weight which one less-CH_2-group. (Stahl, 1969)

The plotting of amount of pyrethin I in relation to peak area ratio of standard calibrating curve (Figure 28) shown an excellent linearlity of response. This was designed to utilize for determination of pyrethrin content *in vitro* culture cells. Figure 29, the topical test, illustrated profile of pyrethrin I contents in callus which was

decreased from first to third developmental stage, it was, may be caused of the mortality of cells when cultures were maintained in the same conditions more longer than the given time.

Quantitative analysis of pyrethrin by HPLC

From the early study, pyrethrins which has been found to be in callus culture (Figure 29). As the problem of unsufficient specimens for GC quantitative determination so the HPLC quantitative analysis was established for determination of pyrethrins contents in suspension cells. HPLC chromatograph in Figure 30 clearly indicated the differences between culture cells and reference standard.

The results of refined pyrethrum suspension cells which was extracted by given conditions (Figure 9) found to gain too much impurities which may be either the part of vegetative extractives or lipids containing in cells with varions types of oils.

Figure 31 shown the analysis of pyrethrin I that found in each suspension cells which was cultured on MS medium supplemented with desired conditions could be found in each developmental stage. The result of this experiment appeared to be as same as reported by Luckner (1980). His investigation on *Nicotiana tabacum* indicated that they could produce nicotin when age of culture approached to idiophase and early declining of cell devision stage.

Chumsri and Staba (1975) found pyrethrin in *Chrysanthemum cinerariaefolium* and *C.coccenieuim invitro.* For Cashyap *et al* (1977) cultivated *Tanacetum cinerariifolium* from seedling but they could not

find any pyrethrins. Zieg *et al*(1983) reported his sucessful investigation in selection of high pyrethrin producing clone which contained 200 - 500 µg/g dry weight in callus.

The amount of insecticidal pyrethrins that can be produced by these said cultures could be as high as 706.07 mg/g dry wt. in suspension culture cells when mornitored with HPLC techniquce, but it appeared only 355.76 mg/g dry weight when detected by GC. This compare unfavorably with field grown pyrethrum flover-heads which produce around dry weight 1.236 g/100 g dry wt (Head 1966, Areekul, 1979, Reynold, 1982)

In comparision of natural flower head, leaf and culture cells which were changed in ratio of constituents after 1 year cultivation (Figure 32,33), it may be noticed similarly to the change of cocaine producing in non differentiated callus but in converse morphenane exited only in differentiated shoots. An occurrence of pyrethrin stability was also attractively observed wheather it was past one year, pyrethrin I content has still be nearly the same (Figure 34).

For the problems of changing in metabolite synthesis, one which was reported in 1981 (Staba and Chung) on cincona culture by undifferentiated tissue, shoot and suspension cells could be use for supporting on these results. Morris and Fowler (1980) found morphinane alkaloid in *Papava somniferum* callus while Koma *et al*(1982) reported that thebaine and coaceine could be produced in callus but morphinan could be found only in differentiated shoot.

Conclusions

Through this study on the Synthesis of Pyrethrins in Tissue Culture of Pyrethrum (*Chrysanthemum cinerariaefolium*Bocc.), the conclusion can be drawn as the followings

Study on establishment of callus and cell suspension culture

The maximum callus initiation and growth can be achieved when a full strength MS medium supplemented with 2,4-D and BA at the ratio of 1 to 3 was applied to the culture when it was cultured under 2,000 lux fluorescent illumination for an 8-hour dark period and maintained at the tempeature of 25±2°C. This condition favours the callus initiation and sustaining growth than the culture condition of a half strength medium cultured in continuous dark environment at 30°C.

In case of cell suspension culture, the establishment of cell culture can also be acheived by replacing 2,4-D recombined with BA and IAA recombined with BA at concentration of 0.25 : 3.0 mg/ml and 2.0 : 3.0 mg/ml, respectively. The others conditions has proved to be the same as previously mentioned.

Study on establishment of pyrethrins extraction and analysis

Maximum yield of pyrethrins extract can be established when petroleum ether was used as an extracting solvent in the Soxhlet apparatus at 60°C for at least 6 hours. The extract quantity was also maximized when a qualitative analysis by TLC with a developing solvent

system of n-hexane-n-heptane-ethylacetate (40:48:12) was applied and the the GC and HPLC operating conditions are as the followings.

GC : 3% OV-17, column temperature of 215^{o}C and flow rate of 30 ml/min.

HPLC : Column packed with C_{18}ODS and MeOH : H_2O (3:1) as a mobile phase were proved to be the satisfactory conditions.

Study on the active ingredient of pyrethrum extract and their constituents in culture cells

When comparing three component perspectives, it was found that Cinerin II appeared to be the main component when culture was age over 1 year and pyrethrin I which was an active ingredient of this insecticidal compound was also appeared in the chromatogram. Cinerin I and Jasmolin I were rather hard to clearly identify in after because of the impurities's disturbance.

Study on effect of growth regulator on pyrethrins production in culture cells

The effect of growth regulator, IAA recombined with BA at the ratio of 2.0 to 3.0 mg/ml was more enhanced on the pyrethrins production in culture cell than the another 2,4-D recombined with BA at the ratio of 1 to 3 mg/ml.