CHAPTER I

INTRODUCTION

Pyrethrum is approved by the Entomological Society of America as the common name for the dried flowers of *Chrysanthemum cinerariaefolium.*, **Bocc.**

Botany

Pyrethrum (*Chrysanthemum cinerariaefolium* Bocc.) is a small perennial herb, with deeply lobed leaves of variable shape and length. This herb is cultivated mainly in Kenya, Tanzania, and Ecuador at an altitude of at least 1900 m above sea level (Appendix A). At this elevation and low temperature, the flower heads (capitula) are borned on branched leafy stems rising from a compact crown of foliage, whereas at higher temperature in lower regions, the plant almost exclusively develops vegetatively (Gnadinger, 1945; Glover, 1955).

Historical review

Throughout the world there is a long-established use of local plants for making insecticidal preparations. More than 2,000 plant species in 170 families possess insecticidal properties (Feinstei, 1952). However, only from a few plants have the insecticides been isolated and their chemistry elucidated. The chemical compounds in the isolation are complex esters (pyrethrins), alkaloids (nicotine, anabasine), or heterocyclic aromatic compounds (rotenoids).

Commercial insecticides of plant origin are few. They include the pyrethrins found in some members of Compositae, like *Chrysanthemum cinerariaefolium*, the commercially important species of pyrethrins, and occasionally to *C. coccineum* Wild (garden pyrethrum), *C. marschalli* Aschers including with *Tagetes erecta*L.(Khanna and Khanna, 1976) and *T. minuta*(Jain, 1977) and others, the rotenoids found in *Derris, Lonchocarpus, Tephrosia* (Leguminosae), the alkaloid nicotine from *Nicotiana* spp. (Solanaceae), and anabasine from *Anabasis aphylla* (Chenopodiaceae).

A comparison of the yields of insecticides from plants and tissue cultures is given in Table 1 (Kudakasseril and Staba, 1988).

Table 1 Insecticides from Plants and Tissue Culture

Insecticide	Species	Source	Content	Reference
Pyrethins	*Chrysanthemum cinerariaefolium*	Flowers	1-2%	Casida (1973)
		Callus	0.023-0.113%	Zieg et al. (1983)
		Shoot cultures	0.050-0.341%	Zieg et al. (1983)
	Tagetes erecta	Flowers	0.9%	Khanna et al. (1975)
		Suspension culture	1.16%	Khanna et al. (1975)
Nicotine	*Nicotiana tabacum*	Leaves	2-5%	Fuell (1965)
		Callus	2.14%	Ohta et al. (1976b)
	Nicotiana rustica	Leaves	5-14%	Fuell (1965)
Rotenoids	*Derris elliptica*	Roots	5-9%	Metcalf (1955)
		Callus with rootlets	0.016%	Kodoma et al. (1980)
	Lonchocarpus utilis	Roots	8-11%	Metcalf (1955)
	Crolalaria burhia	Callus	1.35%	Uddin and Khanna (1979)
	Tephrosia purpurea	Suspension culture	2.8%	Sharma and Khanna (1975)
	Tephrosia vogelii	Roots	1.2%	Sharma and Khanna (1975)
Phytoecdysones	*Trianthema portulacastrum.*	Callus	0.036%	Ravishankar and Mehta (1979)
	Achyranthes sp.	Callus	<0.002%	Hikona et al. (1971)

Among these plant insecticides, the most economically important natural plant insecticides are the pyrethrins.

Pyrethrum as the economical plant

Pyrethrum plants have been cultivated throughout the world, but particularly in Kenya and Ecuador. The term "pyrethrum flower" refers most often to the *Chrysanthemum cinerariaefolium* Bocc., which is the commercially important species for pyrethrins.

Historically pyrethrum goes back to its use as a folk-insecticide in Iran (then Persia) and later to Dalmatia (now a part of Yugoslavia) along the Adriatic Sea.

The commercial production of pyrethrum has shifted with the cataclysmic effects of world conflict. Prior to World War I the principal commercial source of pyrethrum was Dalmatia while cultivation was developing in other nations, especially Japan. The European conflict shut off the Dalmatian production and Japan began to increase its pyrethrum average and became a large degree of the world supplier (Anonymous 1938). Until World War II the economic and military conditions stimulated an increase of production elsewhere. When Japan entered the war on the side of the Axis in December 1941, Kenya, then a British Protectorate and now an independent African nation, rapidly took over the international market outside the Axis countries and is now the largest commercial producer of pyrethrum in the world (Anonymous, 1939).

In 1972 Kenya supplied about two-third of the world's demand for pyrethrum. This dominant position was due in part to the fortuitous combination of the proper growing conditions such as altitude, soil,

rainfall and climate. Other countries in the world also have similar advantages.

In the year 1985/86 it was reported by Mr. Simon Giehuru a senior agronomist at the pyrethrum Bureau of Kenya that Kenya produced about 70% of the world supply of pyrethrum (5,000 tons of dried flowers in 1985/86 season from the Rift Valley and Central Kenya during April/May, 1985 and 2,000 tons of dried flowers in 1985/86 crop years from Western Kenya (Kisii) during August/September, 1985). In 1986/87 the total production was reported to increase to between 9,000 and 11,000 tons (Anonymous, 1985).

Dominant characteristics

Pyrethrins has been designed to be standard active ingredient of insecticide/pesticide by the World Health Organization (WHO). It is rapidly toxic to many insects and has no appreciable effect on insects as a stomach poison but acts by contact. Moreover, it has a much quicker knock-down effect, but less persistent and less stable. Consequently, it is widely used in domestic and agricultural insecticidal sprays and dusting-powders.

The situation in Thailand

Pyrethrum, was first introduced to Thailand by Areekul (1979) under the High Land Agricultural Project, Kasetsart University. Areekul's experiments were done at the Royal Ang Khang Highland Research Station, located in Fang District of Chieng Mai Province in Northern

Thailand. Pyrethrum of Shirayuki variety which was introduced from Japan was used most in experiments. Pyrethrins content determined monthly from flowers of 100 plants samplings were varied from 1.14-2.34%. In clone selection studies, Shirayuki variety I gave higher flower yields and pyrethrins content than Shirayuki variety II. Pyrethrins was also defined to be the standard active ingredient of insecticide/pesticide in Thailand by The Department of Standard Industry, Ministry of Public Helth. Therefore, pyrethrum Shirayuki I which was selected to be the only one of the high pyrethrins producing variety was employed in this study.

Chemistry of pyrethrins

"Pyrethrins" is approved as the designation of the active insecticidal ingredients of pyrethrum (Moore and Levy, 1975).

Pyrethrins are produced commercially by harvesting the flower heads of plants.

1. General information of pyrethrins

1.1 Classification

Primary use : insecticide

Secondary uses : Insect repellant and flushing agent.

1.2 Synopsis

A highly insecticidal extract which is of weak mammalian toxicity. It is rapidly detoxified in the gastrointestinal

tract, and on exposure to sunlight.

1.3 Selected properties.

1.3.1 Physical characteristics

Pyrethrins I and II are refined to pale non viscous liquid oleoresin solutions.

I - b.p. 170°C at 0.1 mm Hg with decomposition.

II - b.p. 200°C at 0.1 mm Hg with decomposition.

1.3.2 Solubility

Water at 20°C virtually insoluble, soluble in hydrocarbons and many other organic solvents.

1.3.3 Stability

Rapidly oxidized and inactivated in sunlight : decomposed by exposure to light with loss of insecticidal activity. Rapidly hydrolysed by alkali.

1.3.4 Vapour pressure

Not known, virtually non volatile at ambient temperature.

1.4 Agriculture, horticulture and forestry

1.4.1 Common formulations

0.2-0.4% dusts, 0.5-1.0% ULV sprays, 0.1-0.5% pressure packs, emulsifiable concentrates to give 0.003-0.015% final pyrethrins content (high volume usage). Most formulations contain a

synergist usually piperonyl butoxide and stabilizers; UV screens are also now being included. Also used at a low concentration, admixed with other insecticides as a "flushing" agent.

1.4.2 Susceptible pests

Effective against a wide range of agricultural and forest pests, including sawfly larvae, (*Lepidopterous caterpillars*), leafhoppers, aphids, beetles, and thrips. Particularly useful where rapid "knockdown" paralysis is required, less effective in killing the target pest unless synergised.

1.4.3 Use pattern

As pre-harvest treatment, applied just before harvest on growing bush and vine fruits, deciduous fruits and nuts, forage crops and vegetables, ornamentals. Spray or dust formulations are used on freshly picked fruits and vegetables in the field, in sprays or dusts fruits, tree-nuts, grains, oil seeds and animal feeds during storage. Aerosols and sprays are used for treating food-handling, processing and storage premises, agricultural premises and households. Used as spray for the control of ectoparasite of livestock (including daily and meat cattle) and poultry. Also used for the control of blowfly during fish drying.

1.4.4 Unintened effects

Pyrethrins are toxic to cold blooded animals : avoid contamination of watercourses. Not phytotoxic.

1.5 Public health programmes.

1.5.1 Common formulations

0.2-0.4% dusts; 0.2-0.4% solutions in kerosene as flying insect sprays; synergists are included in the formulations. 0.5-2.0% shampoos for human and pet usage. Repellant to mosquitos and biting flies : used in insect repellant cream including aerosols and mosquito coils.

1.5.2 Susceptible pests

Active against mosquitos, flies, cockroaches, lice including human body lice, and other public health insects.

1.5.3 Use pattern

Applied as thermal fogs, mists for non-residual control on a repetitive application basis in situations such as kitchens, food stores, factories, etc., where toxic residual insecticides cannot be used. Thermal fogs of 0.05% pyrethrins + 0.40% piperonyl butoxide used for fly control. ULV sprays used against mosquitos, houseflies, and tsetse. Alcohol solutions applied to free water mains of arthropod infestations.

1.6 Household use

1.6.1 Common formulations

0.15-0.30% dusts; 0.15-1.2% "aerosol" pressure packs; 0.05-0.10% sprays; synergists and additional killing agents are often included. Mosquito coil containing 0.15-0.5% (unsynergised)

pyrethrins.

1.6.2 Susceptible pets

Mosquitoes, houseflies, midges, cockroaches, etc.

1.6.3 Use pattern

Aerosol fly sprays use more than half the total production of pyrethrum and mosquito coils about one-third. Used in aerosols and sprays to give rapid knockdown, killing power being increased by use of a synergist. Flushing action useful in expelling insects from hiding places. Mosquito coils protect biting for five to seven hours, depending on mosquito density and ventilation.

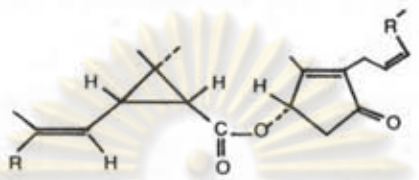
2. Basic structure

The term "pyrethrins" includes the known active principles of pyrethrum flowers which are esters of crysanthemic acid and the corresponding dicarboxylic acid monomethyl ester, also know as pyrethric acid (Moore, 1951).

The publications of La Forge and Barthel (1947), Harper (1949a, 1949b, 1951), Campbell and Harper (1945, 1952), Harper and Reed (1951), Harper *et al.* (1951), Inouye (1951) and Crombie (1953) some twenty to twenty-five years ago focused on the attention current knowledge of the chemistry of pyrethrum.

Since then the great advance in the field of instrumentation, coupled with the discovery of the heterogeniety of the pyrethrolone radical, has advanced the knowledge of pyrethrum chemistry

beyond that known in 1945. La Forge and Barthel (1947) have shown the structure of the active ingredients of pyrethrum, known collectively as pyrethrins, to be esters as represented by the structure shown below (Figure 1).

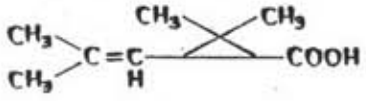


General Formula

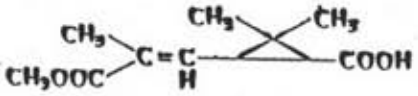
Chemistry of Pyrethrins		Substituent Group	
Fraction	Ingredient	R	R
Pyrethrin I	Pyrethrin I	$H_2C-CH=CH-CH=CH_2$	Me
	Cinerin I	$H_2C-CH=CH-Me$	Me
	Jasmolin I	$H_2C-CH=CH-CH_2-CH_3$	Me
Pyrethrin II	Pyrethrin II	$H_2C-CH=CH-CH=CH_2$	COOMe
	Cinerin II	$H_2C-CH=CH-Me$	COOMe
	Jasmolin II	$H_2C-CH=CH-CH_2-CH_3$	COOMe

Figure 1 Active ingrdients of Pyrethrum

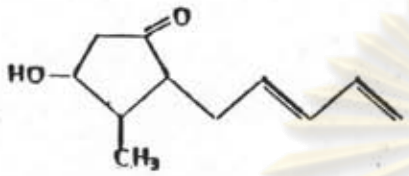
The esters of the pyrethroids are derived from the corresponding acids (Figure 2a) and (Figure 2b) and a five-membered alcohol containing a ketone and a 4- to 5-carbon side chain. For example, the alcohol moiety of the pyrethrum is pyrethrolone (Figure 2c) and that of cinerins, cinerolone (Figure 2d). Chrysanthemic acid may exist in four stereoisomers due to the two asymmetric carbon atoms in the cyclopropane ring. The natural acid has the d-trans-configuration and this has been shown to be more insecticidally active than any of the other isomers or the racemic mixture. Harper and others (1964) have synthesized, separated and optically resolved all of the isomers of this acid.



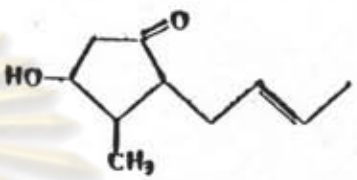
2a Chrysanthemummonocarboxylic Acid



2b Chrysanthemummonocarboxylic Acid



2c Pyrethrolone



2d Cinerolone

Figure 2 Basic structure of Pyrethrins, insecticidal esters.

Pyrethric acid may exist in eight stereoisomers caused by the trans- or cis-configuration of the side chain of the double bond and of the cyclopropane. The natural acid has been shown to have the trans-configuration. As in the case of the monocarboxylic acid, the natural configuration is more insecticidally active than the racemic mixture or any of the three isomers synthesized.

Pyrethrolone and cinerolone have one asymmetric carbon at the 4-position and a double bone in the side chain (R), which is capable of cis-trans isomerism in the 2-position. It is possible, therefore, to have four stereoisomers for each keto alcohol. Katsuda and others (1985) have shown that only the (+) form occurs in the natural esters. Elliott (1964) was unable to identified pyrethrolone C. It is in reality pyrethrolone contaminated with thermally isomerized material. The natural configuration of the keto alcohol portion of the esters is insecticidally more active, as is the case with the acid moiety.

Biogenesis of pyrethrins

1. Acid moiety

1.1 Synthesis of chrysanthemumdicarboxylic acid

Harper *et al.* (1954) established and clarified the stereochemistry and configuration of the d-trans-chrysanthemic acid and the d-trans-pyrethric acid. Crombie *et al.* (1957), following up the Harper's work, concerned themselves with the synthesis of pyrethrins. The report deals with the total synthesis of d,l-cis- and trans-chrysanthemumdicarboxylic acid, d,l-cis- and trans-pyrethric acid and pyrethrin II. This work led to an intensive interest in the synthesis of pyrethrinoids and their component parts.

Inouye *et al.* (1952) reported a novel route of synthesis of chrysanthemumdicarboxylic acid. It was based on the addition of dimethyl diazomethane to the methylmuconic acid ester, the doublebond of d-methylmuconic acid ester to form a cyclopropane ring (Figure 3). The confirmation of the geometrical configuration of the isomeric chrysanthemumdicarboxylic acids with cis-side chain and acylic by-products was obtained by thermal decomposition.

1.2 Synthesis of chrysanthemummonocarboxylic acid

Julia *et al.* (1964) worked out a synthesis of d,l-trans-chrysanthemic acid (Figure 4) by treating the 4-methyl-3-isobutenylvalero-lactone with hydrogen chloride or hydrogen bromide in methanol or ethanol to open the lactone ring; cyclizing the resulting alkyl-5-methyl-3-(1-halogene-methylethyl)-hex-4-enoate into a d,l-trans-alkyl chrysanthemate by treating with an alkali base such as t-butylate,

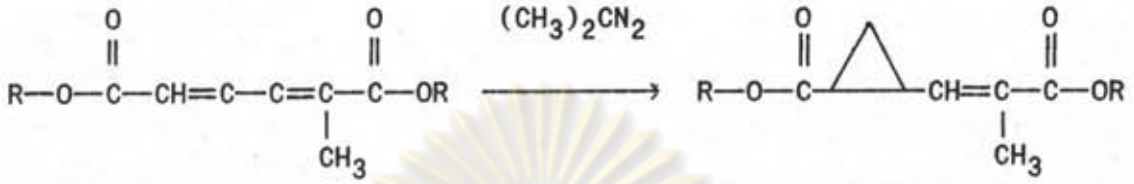


Figure 3 Synthesis of chrysanthemumdicarboxylic acid

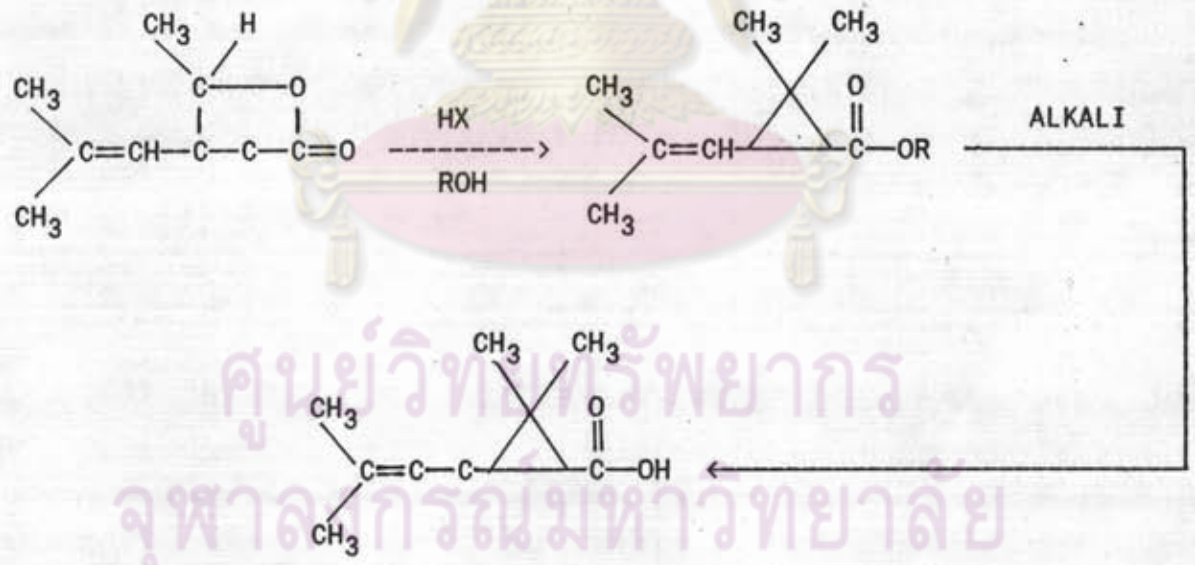


Figure 4 Synthesis of chrysanthemummonocarboxylic acid

t-amylate, amide, or hydride; and converting the ester group of the chrysanthemate into a carboxylic acid group by saponification.

1.3 Synthesis of chrysanthemumdicarboxylic acid from chrysanthemummonocarboxylic acid

Matsui and Meguro (1965) received a United States patent for the production of a d-trans-pyrethric acid. They partially saponify dimethyl trans-chrysanthemum-dicarboxylate with an equimolar quantity of an alkali metal hydroxide and lower alcohol in water; for example, sodium hydroxide in aqueous methanol or ethanol. The mixture is allowed to stand at room temperature or below 60^{o}C for 12 hours to several days. The alcohol is then removed by distillation and water is added and acidification of the equimolar quantity of quinine in acetone. The d-trans-pyrethric acid quinine salt is soluble in acetone but the salt of the isomeric acid is only slightly soluble. Thus, by fractional crystallization of the quinine salts, pure d-trans-pyrethric acid is obtained. Matsui and Yamada (1964) were issued a Belgian patent on another method for the production of pyrethric acid comprising the series of steps as of Figure 5.

2. Alcohol moiety

2.1 Synthesis of pyrethrolone

Crombie *et al.* (1968) were successful in obtaining a superior method to synthesise pyrethrolone by way of a novel Wittig Synthesis which involves the conversion of alkylidene triphenyl phosphoranes into cis-olefins by reaction with aldehydes or ketones. It was completely stereoselective and gave pyrethrolone with an overall

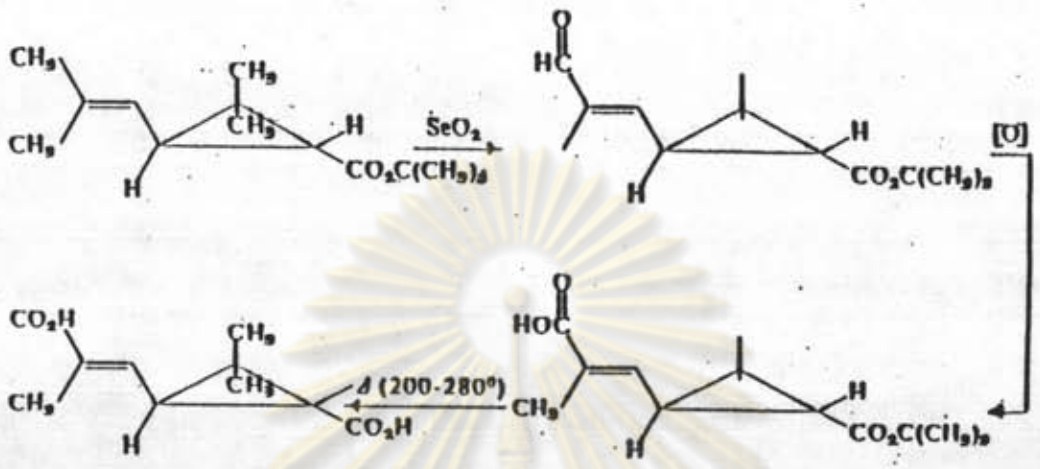


Figure 5 Synthesis of chrysanthemumdicarboxylic acid from chrysanthemummonocarboxylic acid

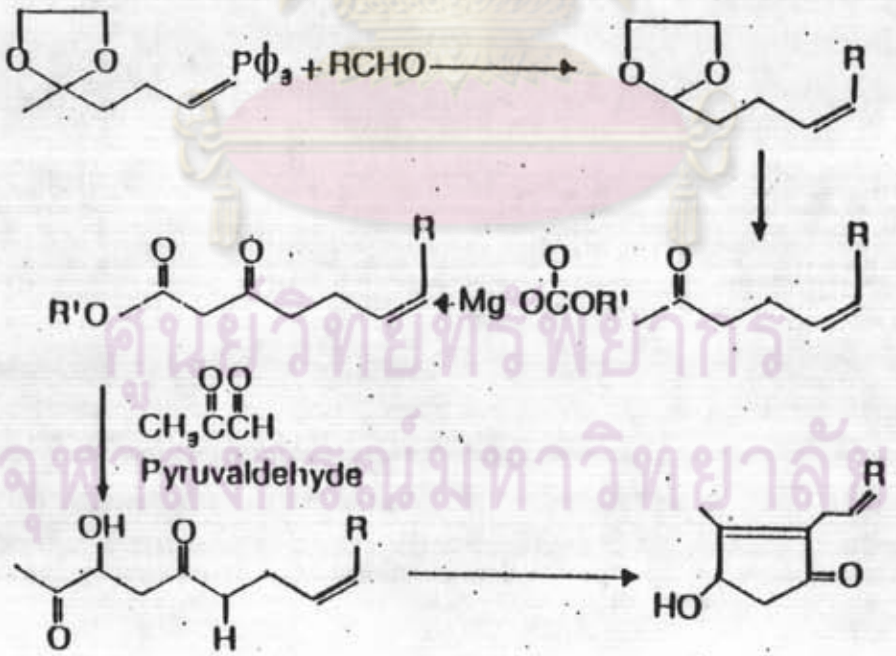


Figure 6 Synthesis of pyrethrolone

yield of 21% (Figure 6).

Plant tissue culture as a unique and valuable research tool

Plant tissue culture is the technique of growing plant cells, tissues, and organs in a prepared nutrient medium and in the absence of microorganisms. There are two main applications of plant tissue cuture. As a unique and valuable research tool, growing plant tissue *in vitro* can help minimize the effect of variables such as environmental factors. Better result can be attained by controlling light, temperature, gas mixtures, and nutrition. Correlative influences, which are working relationships between two or more organs within a plant, can be reduced. Plant tissues can be grown in the absence of artifacts attributable to bacteria, fungi, algae, small organisms, and possibly, viruses. Plant tissue culture can also be employed to preserve valuable germ plasma and as a tool for the plant breeder.

A recent application of plant tissue culture shows the use of this technique for the production of economically valuable chemicals. Plant cells and tissues in the culture can be manipulated so that specific chemicals can be extracted from the cultured tissues or from the medium in which the tissues have grown. In addition, the principle of totipotency, which states that every cell within the plant has the potential to regenerate into a whole plant, can be applied to regenerate plants from cultured cells and tissues. The propagation of valuable economic plants through tissue culture is based on this principle of totipotency.

1. Vegetative propogation of pyrethrum *in vitro*

The application of the tissue culture technique for the rapid multiplication of the pyrethrum plant, has circumvented some of the difficulties encountered in the breeding of this vital plant. (Roest, 1976; Grewal and Sharma, 1978; Florence and Rangan, 1981; Levy, 1981). Often, there is considerable variability associated with the genotype of the plants used as explants and the ease of organ regeneration, e.g. shoot regeneration, root regeneration, *in vitro* is usually related to the ease of vegetative propagation by traditional nursery methods. Conversely, plants which are difficult to propagate vegetatively are also often difficult to regenerate *in vitro*. All these following investigations have been shown to be capable of pyrethrum vegetative propagation.

1.1 Organ source

Roest (1973) used capitulum as source of explant cultivated on basic culture medium containing Difco Bacto-agar (0.6%), Knop's macro- and Heller's microelements (both at half strength), sucrose (2%) and 6-BA (1 mg/l) with fluorescent light illumination about 10 hours dark period at 18°C during the day and 14°C during the night. It was found that two weeks after inoculation a high number of shoot (1-50, with an average of 25 per segment) developed. Further the basal end of the most elongated shoots were treated with 1% β-indoleacetic acid and then transferred to unsterilized soil (a mixture of leaf mould and sand). Consequently, under high relative humidity control within a period of two months, plantlets were produced.

Grewal (1978) reported that the vegetative multiplication of propagules of pyrethrum was acheived through tissue means. Multiple plantlets obtained by using shoot tips cultivated on RT medium containing BA (10^{-5} M) with NAA ($5x10^{-5}$ M) (ca 100 shoots/culture) were grown on sand under intermittent mist and roots appeard within 8 days of planting in 70% of shoots, thus the established plants were successfully transferred to soil.

Mongkolsuk (1984) reported her successful investigation of shoot tip culture cultivated on semisolid MS medium supplemented with either NAA (1 mg/l) and BAP (1 mg/l) or IAA (10 mg/l) and BAP (1 mg/l) provided 34 and 44.6 shoots respectively after 5 weeks of inoculation. The formation of shoot was also obtained by culturing shoots on medium containing only IAA (5 mg/l) and subsequently transferred to non-sterile mixture of burnt-rice-husk and sand (1:1) which provided the highest survival percentage.

1.2 Growth regulators

The single most important factor that determine an organ formation in tissue culture is the relative quantities of auxin and cytokinin (Skoog and Miller, 1957). They appear to be a universal control, by the relative levels of auxin and cytokinin, of a regulatory mechanism which leads to organogenesis within the cells and tissues of plants. Pyrethrum leaf blade and leaf petiole produce callus when exogenously supplied with auxin and cytokinin of approximately the same molar concentration (Vesprasit, 1988, 1989). If the auxin level is raised relative to the cytokinin concentration, roots are induced. Conversely, when the auxin concentration is lower than that of the

cytokinin, shoots are formed on the pyrethrum tissue (Pal and Dhar, 1985).

Karki (1979) also suggested that the *in vitro* method of propagation from shoot tip involves three main steps: first, initiation of auxillary buds and their multiplication through subculture, using cytokinins in the culture medium; second, rooting the shoots multiplied in first step with or without auxin; and third transfer of the plantlets to the field for establishment. His investigation was confirmed by using shoot tips cultured on MS medium containing BAP (5 mg/l) and IAA (0.5 mg/l) obtained a mass of multiple shoots within 12 weeks after inoculation. For rooting, the multiple shoots were removed and suddenly treated in IAA (100 mg/l) for 5 min. for root induction before transplanting to the field.

Wambugu (1981) had succeeded to establish multiple shoots formation through axillary bud proliferation. Shooting was successfully induced on MS medium containing only cytokinin (6-BA).

The synnergism between coconut water and auxin which was reported by Steward and Shantz (1954) has been found to be, in part, due to an cytokinin/auxin synergism.

2. Establishing callus culture

The most common cultured plant tissue is callus which is wound tissue composed of differentiated, highly vacuolated and unorganized cells. Callus cultures are usually maintained in darkness,

and 2,4-D is frequently added to suppress organogenesis.

2.1 Organ source

Jain (1977) reported that hypocotyl and epicotyl callus of *Tagetes minuta* had been shown to be capable of pyrethrins synthesis. Explants were cultured on 1% agarized Murashige and Skoog's revised tobacco medium (RT) supplemented with 2,4-D (1.0 mg/l). The experiment performed at 26 ± 1°C under normal room light conditions. Subculture were made every 6-8 weeks and later fresh tissue were harvested for Thin-layer chromatographic (TLC), Spectrophotometric and Gas Chromatographic (GC) analysis.

In the same year, another report described the use of callus derived from 6-week old seedlings of *Tanecetum cinerariaefolium* contained pyrethrins when cultured statically at 27°C under a fluorescent light of ca 1500 lux intensity, on a modified Murashige and Skoog's medium supplemented with 2,4-D (0.5 mg/l), kinetin (0.75 mg/l) and casein hydrolysate (1 g/l) solidified with Agar (0.7%) with frequent sub-culturing. This condition could provide a 4 kg sample of the callus for pyrethrin TLC and Gas Liquid Chromatography (GLC) analyses (Cashyap *et al.*, 1977).

The results obtained by Zieg *et al.* (1983) revealed that callus derived from either all parts of flower head, e.g. disc florets; ray florets; achene; receptacle or leaf, e.g. leaf epidermis, leaf mesophyll including peeled leaf apparently contained pyrethrins.

In 1985, a report published in Pyrethrum Post, an official publication of the pyrethrum Bureau, stated that callus

initiation from leaf and petiole explants could be succeeded within 18 days of inoculation. This successful investigation was only achieved in few treatments with MS basal medium containing only cytokinins at different concentrations and also in treatments with the combination of auxin and cytokinin. The response of explants in the primary medium and calli in the secondary medium were also observed and further summarized in both color and pattern differences. The obtainable color were yellowish green, cream-white and brown callus, meanwhile, the pattern of calli were compact, friable nodular and loose callus. In addition, the cytological studies of calli and root-tips were reported. Mitotic irregularities were observed from those fixed tissues and micrographs taken. Furthermore, the results of this investigation confirmed that pyrethrins could be measured in tissues grown on those treatments.

Kueh *et al.* (1985) also found that leaf laminae and leaf petioles calli could be initiated by growing tissue on solidified MS medium supplemented with 2,4-D (0.5 mg/l), kinetin (0.75 mg/l) and casein hydrolysate (1.0 g/l). Such calli were also found capable of producing pyrethrins.

2.2 Factors influencing callus initiation and establishment.

2.2.1 Media formulations

Although whole plants have simple requirements for the growth and cell development a callus culture has more complex needs and is seldom autotropic. That is, plant tissue *in vitro* requires the usual macro- and microelements supplied in hydroponic culture (Murashige and Skoog, 1962; Gamborg, 1975). In addition, other nutrient

such as source of bound carbon and vitamins are necessary (Huang and Murashige, 1977). Isolated plant cells and tissues frequently require the addition of vitamins and plant growth regulators that *in vivo* are synthesized by one part or organ of a plant and a plant transported to another part where they are metabolized (Skoog and Miller, 1957; Street, 1973). Salt requirement for cultured plant tissues was reported by White (1934), Gauthert (1939), Murashige and Skoog (1962), Gamborg *et al.* (1968).

The salt requirement for cultured pyrethrum plant tissues was first reported that seedlings were caltured on a full strength MS medium (Khanna *et al.*,1976). Jain (1977), Cashyap *et al.* (1977), and Kueh *et al.* (1985) reconfirmed by the use of full strength MS medium for culturing pyrethrum plant tissues.

Meanwhile, Zieg *et al.* (1983) succeeded to culture disc flowers on a half strength MS medium. The response of explants in the primary medium was satisfied and consequently subcultured to the full strength MS medium for callus maintenance.

In the case of growth regulator requirement for cultured pyrethrum plant tissues, Zieg *et al.* (1983) reported that high pyrethrins producing callus derived from various parts of pyrethrum tisses required different concentrations of growth regulators. A callus derived from leaf required a lower of 2,4-D (0.05 mg/l) recombined with BA (3.0 mg/l) than a callus derived from a young bud. Kueh *et al* (1985) suggested that callus derived from leaf and petiole could be successfully initiated on MS medium containing 2,4-D (0.5 mg/l) and Kinetin (0.75 mg/l). In addition, Pal and Dhar's results confirmed that

the growth regulators requirment for callus initiation and establishment in difference level depended upon the organ source of the explant.

2.2.2 Culture environment for callus initiation and establishment

Light is one of the most essential factors for establishing callus culture. Plant tissue cultures are not photosynthetically efficient and therefore, generally are not autotrophic (Gamborg, 1986). Nevertheless, the influence of light on morphogenetic processes within the cultured tissue should be discounted. Hence, when maintaining callus cultures, the tissue should be maintained in darkness to avoid morphogenesis. Pal and Dhar (1985) observed that rapid callus initiation occurred under the photoperiodic condition and the callus proliferation also increased by three-folds under the photoperiodic condition in comparision to those grew in the dark.

Cashyap (1977) reported his success of establishing callus under 1,500 lux of the continuous fluorescent illumination. Temperature, another essential factor, has been proved as a requirement of the initiation and establishment of callus culture. Temperature in the range of 25-27^{o}C is normally employed for *in vitro* culture (Skoog, 1944; Steward, 1954; Street, 1973; Gamborg, 1975; Tabata, 1977). The optimum temperature for the growth of pyrethrum callus culture has been reported by many researchers (Khanna, 1976; Cashyap, 1977; Kueh, 1985; Pal and Dhar, 1985;Zieg, 1985) that the range of 25-27^{o}C is also suitable for callus initiation and establishment.

3. Optimum volume of medium per culture vessel

The optimum amount of medium for each tissue and vessel type should be tested carefully. Too much medium can adversely affect tissue growth. Conversely an inadequate quantity of medium can slow the growth rates of tissue (Staba, 1982).

4. Sterilization of medium

Plant tissue culture medium are sterilized by autoclaving or by filter sterilization. The most common technique is to sterilize the media, which has previously been dispensed into culture vessels, at 121^{o}C , pressure 15 lbs/inch^{2} for 15 min. in an autoclave. When autoclaved, sucrose could combine with other media constituents and caused a non-enzymatic browning which is toxic to cultured plant cells (Peer, 1971). Media constituents which are heat labile (e.g. gibberellic acid) (Van Bragt, 1971), can be filtered, sterilized and added to the remainder of the constituents which have been autoclaved and maintained at 40^{o}C to prevent agar solidification.

The use of cell culture technique for the production of economically valuable products

Over the past years there has been increasing interest in the use of plant cell culture as industrial biosynthetic sources of useful secondary products. According to. previous investigations of many researchers (Chan and Staba, 1965., Kaul *et al.* 1967,1969; Veliky, 1972; Stohs, 1972; Hahlbrock, 1972; Austin and Brown, 1973; Bonlanger, *et al.*, 1973; and Kurz *et al.*, 1980), cell cultures were potentially valuable for the study of the biosynthesis of secondary metabolites and may also

eventually provide an efficient means of producing commercially important plant products.

1. Techniques of cell suspension culture

1.1 Free cell and aggregates of cell formation

Once friable callus has been obtained the tissue can be transferred to a liquid medium. If vigorious agitation and aeration is then applied, a suspension of free cells and aggregates of cells can be obtained. Kueh *et al.*(1985) obtained cell suspension culture by using friable callus derived from leaf and petioles cultured in liquid MS medium containing ^{14}C-labelled chrysanthemic acid. His work summarised that chrysanthemic acid was accumulated largely as a water soluble glucoside ester in the pyrethrum cell cultures.

1.2 Batch cultures on plateform shakers

As mentioned above, the formation of free cell and aggregates of cell and the successful establishment of the suspension culture depends upon the initial callus being friable. It may be necessary to use an appropriate level of GR in the callus culture medium to achieve the necessary friability of the callus. The culture medium used for the suspension culture is usually based upon which maintains good growth of callus; it may, however, be necessary to modify this medium (particularly its GR content) to achieve a high growth rate and good cell separation in liquid medium. Platform shakers are widely used for an initiation and serial propagation of plant cell suspension cultures. They should have variable speed control (30-150 rpm) and the stroke (throw) should be in the range of 2-4 cm orbital motion. Lamport

(1969) reported that an appropriated speed control for culturing suspension cells of higher plant should be 80-120 rpm. The results of Short *et al.* (1969) on the growth in culture of *Acer pseudoplatanus* cell suspensions also supported the above finding.

The Suitability of cell cultures for secondary metabolites biosynthesis

On the first biosynthetic examination it would appear that cultured cells offer several advantages over intact plants. They are relatively easily grow and can be kept under strictly controlled nutritional and environmental conditions, hence, the uncertainities of climate and soils can be avoided. They are also cultured aseptically which eliminates the problems associated with contamination by microorganism. Further, cultures often provide simpler and more convenient experimental systems than intact plant. In particular, cultures grown in liquid media offer a very effective way of incorporating precursor materials which are often difficult to administer to the entire plant. Finally and perhaps the most attractive feature is that the technology is now available a large scale production of plant cell suspensions in batch cultures, chemostats and turbidostats.

Pyrethrins, one of the secondary products which have been identified in tissue cultures, are derived from pyrethrum. At present, the prohibition of using DDT and many other highly toxic insecticides in many countries has resulted in an increasing demand of pyrethrins in the world markets. Pyrethrum, however, has limited sources of production because of its specific requirement of limitic conditions and thus prevailing only in certain areas of some countries.

Also, it was clear from the published papers reported by many workers (Khanna, 1976; Cashyap, 1977; Jain, 1977; Zito, 1982; Zieg, 1983; and Kueh, 1985) that the potentialities of the callus tissue and cell suspension to produce pyrethrins even any types of explant are derived from pyrethrum.

In this present study, established cultures were derived from leaf and petiole of Shirayuki I variety provided from the High land Agricultural Project, Kasetsart University. The observations provided evidence that the productivity in cell or tissue culture may be related to the productivity in the natural plants in another way. Further, in order to have comparable results at all these samples (callus, cell suspension and shoot cultures' extracts), it is essential to evaluate which culture techniques and specimen preparation techniques would be most ideally suited for the synthesis of Pyrethrins and for the analytical study respectively. Consequently, the following techniques to induce shoot tip culture, callus cullture, and cell suspension culture were employed.

Objectives

1. The present study is designed to establish factors influencing callus initiation, callus and cell suspension culture.

2. Study on the cytological status of callus and suspension cells in comparison with root tip.

3. Study on the pyrethrins extraction and product analysis.

4. Study on types and patterns of pyrethrins synthezised from different cultures, e.g. shoot tip, callus, and cell suspension culture.

5. Study on factors influencing pyrethrins synthesis in cell culture.