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The Production of Pyrethrins by Plant Cell and Tissue Cultures of *Chrysanthemum cinerariaefolium* and *Tagetes* Species

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Abstract: Pyrethrins, the most economically important natural insecticide, comprise a group of six closely related monoterpene esters. The industrial production is based on their extraction from *Chrysanthemum cinerariaefolium* (Pyrethrum) capitula. The world production of natural pyrethrins still falls short of global market demand stimulating the research in *in vitro* production as an alternative to conventional cultivation methods. The different biotechnological alternatives such as callus cultures, shoot and root cultures, plant cell suspension cultures, and bioconversion of precursors by means of enzymatic synthesis or genetically engineered microorganisms, as well as the progress achieved in methods for the identification and quantitation of insecticidal compounds have been reviewed. Although technology for plant cell culture exists, industrial applications have, to date, been limited due to both the low economical viability and technological feasibility at large scale. Bioconversion of readily available precursors looks more attractive, but more research is needed before this technology is used for the industrial production of pyrethrins.

KEY WORDS: cell and organ cultures, bioconversions, genetic engineering, economical viability, identification and quantification methods.

I. INTRODUCTION

Every year insects cause substantial losses in agriculture and forestry (Metcalf and Metcalf, 1993). They consume about 30% of food grown and about $6000 million is spent annually protecting crops from the havoc they cause. In addition, insects transmit many human and animal diseases. Diptera are vectors of malaria, filariasis, schistosomiasis, trypanosomiasis, and onchocerciasis, which debilitate and kill people and domestic animals on a vast scale. In the home, human and animal lice, fleas, bugs, and ticks cause much irritation, and their bites can spread devastating bacterial diseases such as bubonic plague (Busvine, 1993). Insects pests thus have to be controlled, as benevolently as possible, and with minimum impact on the environment and on mammals.

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Various insecticides and procedures for insect control have been developed and used. The main commercial products are chemical insecticides such as (1) synthetic compounds: pyrethroids (namely, Deltamethrin and Allethrin), organochlorine (DDT and HCH), organophosphorus (Dimfox, Tabun and Sarun), and carbamate (Carbanyl and Carbofuran), and (2) insecticides that affect insect growth by disruption of the moulting of insect larvae (diflubenzuron and chlorfluazuron). The accumulation of "hard" insecticides in the environment with resultant problems of toxic residues, resistance of insects, resurgence of new insect pests, contamination of the agroecosystem, contamination of air, water, and soil, and many other problems, prompted the search for more environmentally friendly methods of controlling insects, such as the use of biodegradable natural insecticides (pyrethrins, nicotine, and rotenone) and biological control agents, for example, semochemicals (Pickett et al., 1991), including pheromones and host recognition compounds (Pickett, 1988), antifeedants (Ley et al., 1993), *Bacillus thuringiensis* (Gill et al., 1992), and neuropeptides (Elliott, 1995).

Pyrethrins, widely used natural insecticides, offer all the advantages of chemical compounds, that is, rapidity of action, activity against a broad range of insects, and low costs, and because of their rapid biodegradability some of the advantages of biological agents such as weak development of resistant strains and low toxicity for mammals. The advent of stricter environmental legislation and the mounting industrial research and development costs of new chemical insecticides have encouraged their use (Van Latum and Gerrits, 1991). Pyrethrins are presently extracted from the flowers of *Chrysanthemum cinerariaefolium* vis. high-productive clones. In the last few years, because of a fall in African production, worldwide demand has exceeded supply, causing an increase in their market price and encouraging research to improve yields and develop alternative methods of production. In the last decade, progress has been obtained in the isolation of *Chrysanthemum cinerariaefolium* vis. high-productive clones derived from selected genotypes and their multiplication by tissue culture. Our purpose in this review is to summarize the progress made in the growth and production of pyrethrins by plant, cell, and tissue culture and to evaluate the usefulness of biotechnological processes for their biosynthesis. The major factors influencing the production of pyrethrins and the economic viability are also discussed.

II. PYRETHRINS

A. Chemical and Biosynthetic Aspects

The pyrethrins are a set of six structurally close monoterpene esters produced by esterification of two monoterpenic acids (chrysanthemic acid and pyrethric acid) with three ketone alcohols (pyrethrolone, cinerolone, and jasmolone). The chrysanthemic acid esters are called pyrethrin I, cinerin I, and jasmolin I, respectively, and are collectively known as the pyrethrins I fraction, whereas the pyrethric acid esters, that is, pyrethrin II, cinerin II, and jasmolin II, form the pyrethrins II fraction (Figure 1, Jovetic and De Gooijer, 1995). The pyrethrins are synthesized by the pathway depicted in Figure 2. Chrysanthemic acid is a monoterpene formed by the isoprene pathway from acetate via mevalonic lactone (Godin et al., 1963). The conversion of radiolabeled acetate and mevalonate into pyrethrins I has been accomplished using cut flower stems and isolated achenes (Pattenden and Storer, 1973). The addition of labeled chrysanthemate to dissected achenes shows it is incorporated into both pyrethrins I,

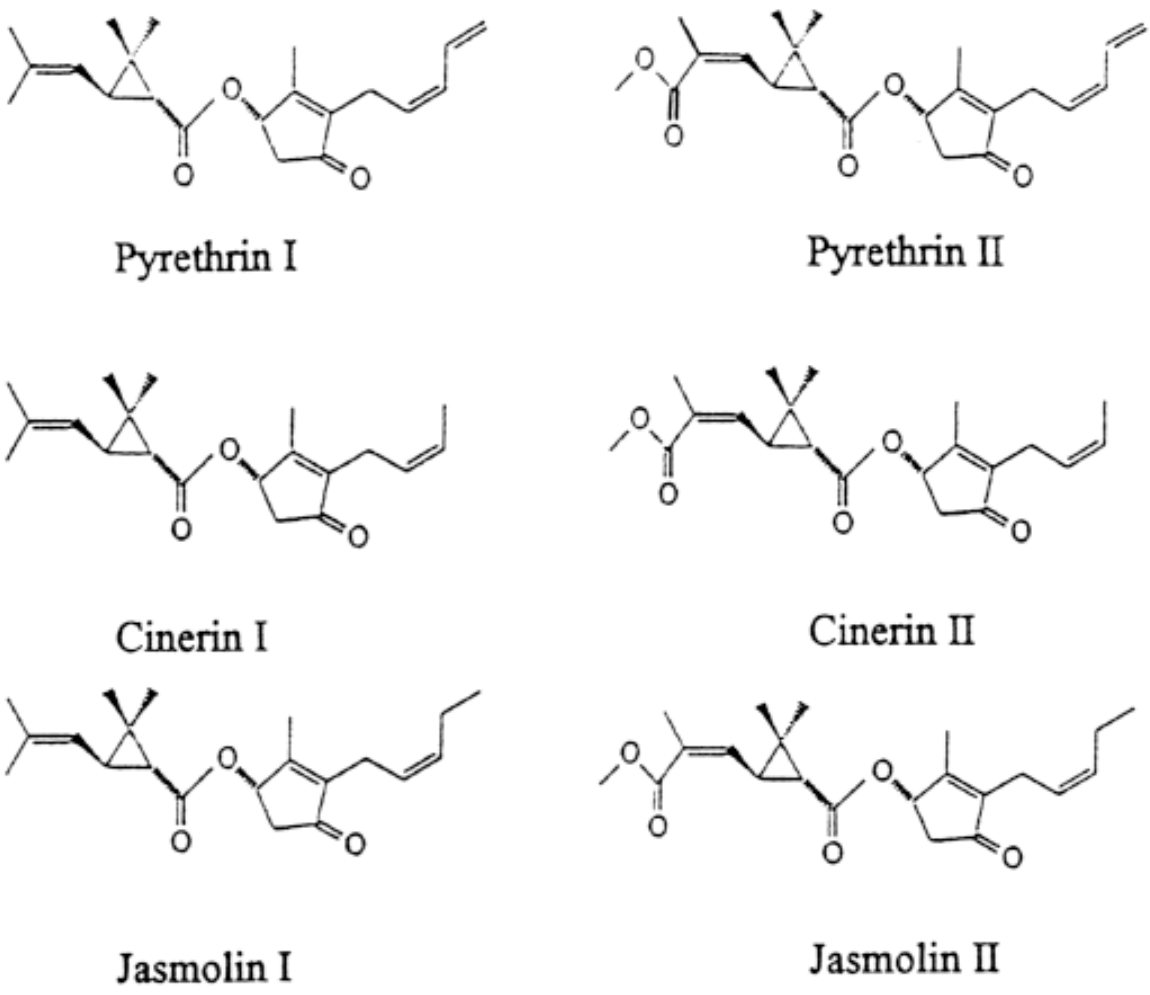

FIGURE 1. Structure of natural pyrethrins.

pyrethric acid, and pyrethrins II, thus indicating that chrysanthemic acid serves as precursor for pyrethric acid (Abou Donia et al., 1973). The incorporation of [1-^{14}C]-isopentenylpyrophosphate into pyrethrins I and chrysanthemyl alcohol has been achieved in a cell-free homogenate system prepared from fresh flower buds (Staba and Zito, 1985). This study established chrysanthemyl alcohol pyrophosphate as the most probable intermediate in biosynthesis. The rethrolone portion of the pyrethrins has been shown to be derived from acetate, but no intermediates have been detected. The acetate is thought to be incorporated via the polyketide pathway (Zito, 1994). A molecular key has been suggested: 12-oxophytodienoic acid results from linolenic acid 13-hydroxy-peroxide (Crombie and Morgan, 1991; Crombie, 1995), which form jasmonic acid (Demole, 1982), which in turn yields pyrethrolone and jasmolone.

B. Sources

Pyrethrins have been identified in different species of the Asteracea (ex Compositae) family: *Calendula officinalis*, *Chrysanthemum cinerariaefolium*, *Chrysan-*

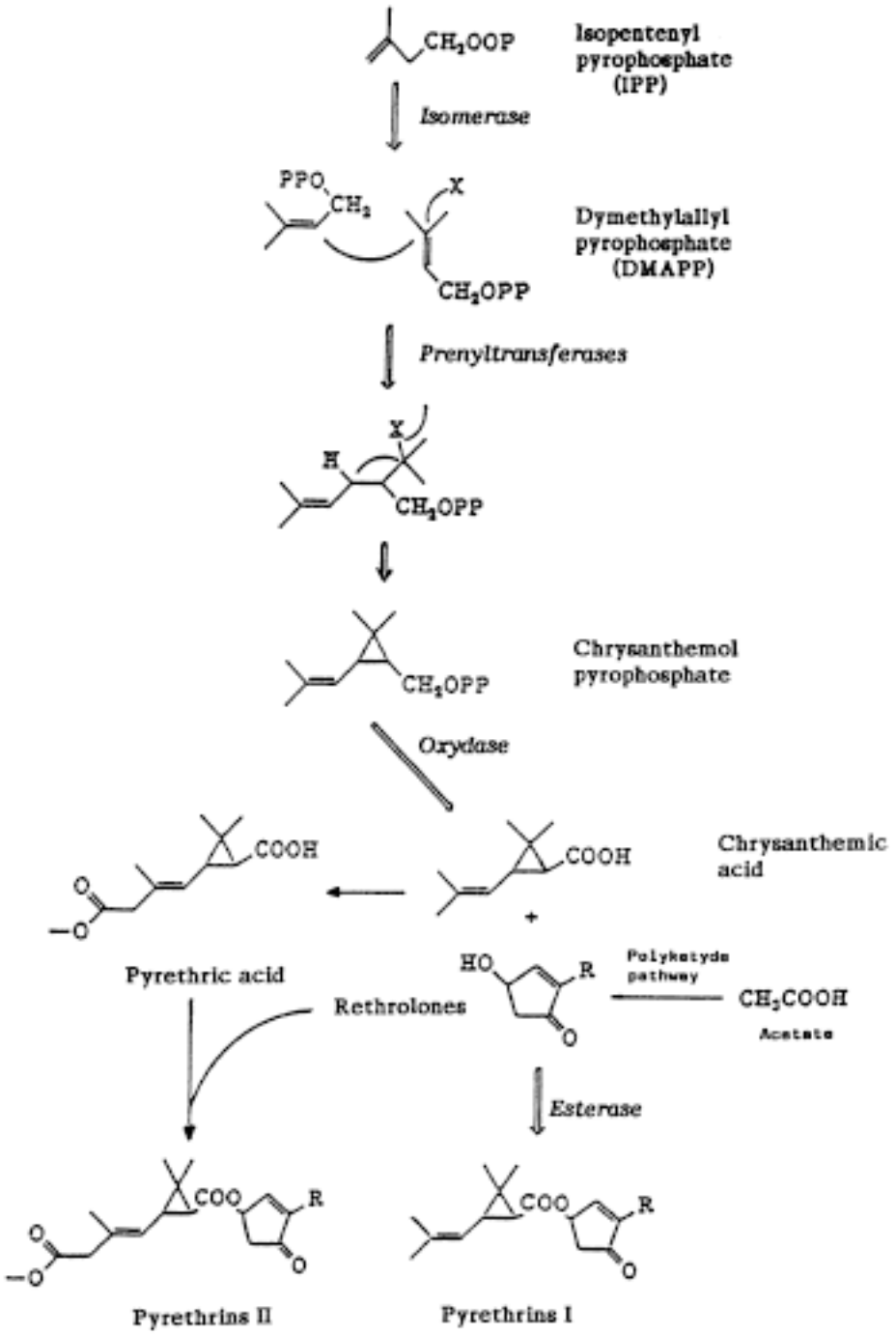

FIGURE 2. Pyrethrin biosynthesis pathway.

themum coccinum, *Tagetes erecta*, *Tagetes minuta*, *Zinnia elegans*, *Zinia linnearis*, etc. (Kudakasseril and Staba, 1988).

The commercial plant, *Chrysanthemum cinerariaefolium* vis., also known as *Dalmatian pyrethrum,* is the principal source of pyrethrins. Dalmatia (a region of ex-Yugoslavia) remained the main source of *Chrysanthemum cinerariaefolium* until the First World War, after which Japan took over as

the principal producer. By 1941, Kenya overtook Japan as the main world producer and, following the outbreak of the Second World War, Japan ceased to be a significant pyrethrum supplier (Moore and Levy, 1975). Between 1971 and 1990, the introduction of synthetic pyrethroids on the insecticides market depressed world demand for pyrethrins, thereby reducing plant cultivation. There has been a recent shift in consumer demand back toward natural products, which has reversed this trend.

C. Biogenesis, Use, and Market Volume

Pyrethrins are located in all the aboveground plant parts, but the achenes of the flower heads contain approximately 94% of the total pyrethrins (Brewer, 1973) in the inner secretory ducts (Head, 1966, 1973). The flower head accumulates pyrethrins to the extent of 0.8 to 2% of the flower dry weight (Davies, 1985). Clones able to synthesize more than 3% pyrethrins are now used by companies such as SANOFI Chimie (France). The content in pyrethrins depends on genotype, picking interval, flower maturity, climate, and drying methods. In parallel, the yield of fresh flower is determined by the genotype of the selected clone, soil, climate, plant diseases, and pests (Jovetic and De Gooijer, 1995). Pyrethrin yields and quality of plants extracts are determined by pyrethrin content, flower yield, and the pyrethrins I/pyrethrins II ratio. Pyrethrins I are known to have a good knock-down action, while pyrethrins II have a better kill effect (Elliott, 1989). Pyrethrin I and II are highly insecticidal, and cinerin I, II and jasmolin I, II, are much less bioactive (Zong-Mao and Yun-Hao, 1996). Usually, a typical commercial extract can have equal amounts of pyrethrins I and II (Crombie, 1980), but ratios ranging from 0.47 to 3.5 have been observed in different breeding lines (Head, 1967; Bhat, 1995).

The natural pyrethrins have all the qualities of an ideal pest control agent. They are effective against a broad range of insects with little development of resistant strains (Mrack, 1973). *Chrysanthemum cinerariaefolium* extracts have been demonstrated to be effective on *Lygus* spp., *Leptinotarsa decemnileata*, *Pieris rapae*, *Aspodydia* spp., *Empoasca devastans*, *Leucinodes orbonalis*, *Ophiomyia reticulata*, *anthonomous rubi*, *Earias fabia*, aphids, flies, beetles, cockroaches, ants, mosquitoes, grasshoppers, and numerous caterpillars, mites, thrips, and moths (Van Latum and Gerrits, 1991). They act rapidly in two ways, knock-down and kill. 'Knock-down' occurs within a few minutes affording early paralysis of insects, whereas 'kill' occurs several hours after the treatment. The action site of pyrethrins has been found at the neuronal voltage-sensitive sodium channel (Kueh et al., 1985). They pose little risk to humans and other mammals as determined by toxicological tests (Environmental Protection Agency, 1989; Shoenig, 1995), and their repellency may be more important than the killing effect when protecting food (Crombie, 1980). Pyrethrins, being unstable in air and light, rapidly lose their insecticide activity (Allan and Miller, 1990). Thus, the non-persistence of pyrethrins makes them widely acceptable as safe and environmentally innocuous alternatives to other "hard pesticides" (Otieno, 1983). Pyrethrins are suitable as an all-purpose crop protection and disease vector control agents when properly formulated with antioxidants (tannic acid), stabilizers (hydroquinone), and synergists (piperonyl butoxide, sesamin, mysristicin). In these conditions, they are still economically viable insecticides (Balandrin and Klocke, 1988). In the mid-1980s, $20 million was the estimated value of the annual U.S. market, with a wholesale price of $300/kg (Balandrin and Klocke, 1988). Market size has since increased substantially and is estimated to be as high as $400 million a year; the wholesale price of

flower extracts, on a basis of 100% pyrethrin content, was reported to be 413 $/kg (Shand, 1992). The global market volume is more than 12,000 tons of dry flowers per year (Verpoorte et al., 1993), while the world demand for dry flowers remains at 20,000 tons a year (Sihnya, 1992). Conventional production of pyrethrins still falls short of world requirements (Magnier, SANOFI-Chimie, personal communication), despite an increased production of *Chrysanthemum cinerariaefolium* flowers and pyrethrins, especially in Australia (Table 1).

D. Extraction and Analytical Procedures

Pyrethrum flowers are harvested and dried in the open air to avoid fermentation and pyrethrin losses. The dried flowers are ground and extracted with hexane or another suitable solvent (e.g., petroleum ether). More recently, supercritical CO_2 has also been tested for the extraction of pyrethrins for pharmaceutical purposes. Undissolved plant matter is filtered out and the solvent is flashed off to leave a crude oleoresin that typically contains about 30% pyrethrins.

Numerous analytical methods have been used to identify and estimate the content in pyrethrins. The reference quantification method is the AOAC/titrimetric procedure. This quantifies pyrethrins I and pyrethrins II groups after saponification and quantitation of chrysanthemic and pyrethric acid with the mercury-reduction procedure. This method has numerous drawbacks. It requires long saponification steps, it is unsuitable for the analysis of residues, and it does not quantify the six pyrethrins independently (Carlson, 1995). However, it is the reference method used to quantify commercial products and serve as a standard for other analytical methods. Gas liquid chromatography (GLC) offers the advantages of speed and reduced handling, but suffers from the drawback of pyrethrins II degradation by the high temperature. Usually, quantification by GLC is based only on pyrethrins I (Carlson, 1995). Numerous methods for quantification of pyrethrins by GLC in various samples were developed (Class, 1991; Nakamura et al., 1994; Nguyen et al., 1998).

Currently, the breeding programs of *Chrysanthemum cinerariaefolium* include the multiplication of high-productive cell lines selected on the basis of both chemical and agricultural characteristics. Valuable chemical characteristics included the yield in pyrethrins and the insecticide activity of the selected clones. This last parameter is related not only to the pyrethrins I/pyrethrins II ratio (as conventionally estimated) but also to the relative proportions of the different esters inside the same group. Thus, it is important to have an analytical method that will assay each pyrethrin independently. Normal (McEldowney and Menary, 1988; Bushway, 1985) and reversed (Kamau, 1990; Wang et al., 1997 to 1999) phase HPLC has proved a valuable method for pyrethrins analyses. The technique offers the advantages of high resolution and reduced handling without risk of pyrethrin degradation.

TABLE 1
Australian Production of Pyrethrins

	Annual production (tonnes)			
Product	**1989**	**1990**	**1991**	**1992**
Dry flowers	350	800	1.30	2.00
100% pyrethrins	5	10	14	21
20% pyrethrins (refined product)	25	50	70	105

UV is the most often used detector, although refractive index and fluorescence have also been employed. A recent technique is an adaptation of chromatography using supercritical fluids (SCF) as the carrier phase (Wieboldt et al., 1989; Lubke, 1991). For the qualitative determination of pyrethrins, the coupling of chromatography procedures (GLC or HPLC) with mass spectrometric detection has been used (Nikiforov and Kohlmann, 1983; Rajasekaran et al., 1993). With regard to detection by GLC, electron-capture detection is most frequently selected in the pyrethrin analysis. With this detection method the detection limit is ranged in nanogram-to-picogram level (Zao-Mao and Yun-Hao, 1996; Berger-Preiß et al., 1997). A new identification and quantitation method has been developed recently by us using direct infusion of extract in the mass spectrometer, a tandem mass spectrometry method (MS/MS) was used to determine and to quantify the individual components of pyrethrins in the plant extracts (Barthomeuf et al., personal communication). The method has the advantage to being simple and accurate and not requiring extensive clean up procedures. The detection is highly specific, and the six pyrethrins are assayed without prior separation. The limit of detection is 0.3 ppb (instead of 0.3 ppm with classic HPLC methods). The proposed procedure is a valuable tool for routine analyses of residues and rapid monitoring of each pyrethrin content during selection of high-productive clones or cell lines and could replace the AOAC method for quantitation of standards used for routine analyses of flower extracts by HPLC.

III. BIOTECHNOLOGICAL PROCESSES

The commercial importance of pyrethrins and the unreliability of the supply of plant dry flowers have promoted the exploitation of tissue culture as a way to rapidly multiply high-yield plants producing the pyrethrins and *in vitro* production of pyrethrins. Besides micro-propagation, the cell and tissue culture derived from selected lines may also be used as an alternative raw material for extraction of pyrethrins.

A. *In Vitro* Multiplication

Either splits or shoot cuttings generally achieve vegetative propagation of *Chrysanthemum cinerariaefolium*. However, these propagation methods result in a low multiplication rate. An additional problem is the high vulnerability of plants to attack by the root-knot nematode (*Melodogyne hapla*), and the splitting of infected stock plants, which results in a contaminated clone. In addition to offering increased rates of propagation and pathogen-free plants, *in vitro* clonal multiplication facilitates asexual multiplication of clones that have desirable characteristics. In addition, the plant biotechnology phase is integrated into established plant breeding and crop practices. The propagation of *Chrysanthemum cinerariaefolium* has been well described (Bhat, 1995) (Table 2).

Generally, shoot tips and/or axillary buds are used as explants; they are surface sterilized and placed on a growth medium for production of adventitious shoots. The growth medium is the Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with various auxin and cytokinin concentrations. After 4 to 6 weeks, the adventitious shoots are subcultured on the propagation medium. This cycle is repeated until the required number of plants has been obtained. Levy (1981) has developed a successful mass propagation technique with theoretical production of 62,000 adventitious shoots per month, giving 930,000 plants.

Regeneration of *Chrysanthemum cinerariaefolium* plants from callus cultures has been reported (Pal and Dhar, 1985; Paul et

TABLE 2
The Main Published *In Vitro* Propagation Programs for *Chrysanthemum cinerariaefolium*

Authors	Explant	Medium	Hormones	Remarks
Roest and Bokelmann (1973)	Flowers	Knop	1 to 10 μ*M* BA 6 μ*M* AIA	Multiple shoots vitroplants
Grewal and Sharma (1978)	Shoot tips	MS	1 to 10 μ*M* BAP 5 μ*M* AIA or ANA	Multiple shoots Vitroplants
Cashyap et al. (1978)	Shoot	MS	0.5 mg.l^{-1} 2,4 D + 0.75 mg.l^{-1} Kin	Callus
			1 mg.l^{-1} ANA	Shoot
Wambugu and Rangan (1981)	Axillary buds	MS 0.9 μM BAP	0.1 μ*M* BAP Multiple shoots	Shoot
			1.1 μ*M* ANA	Vitroplants
Zito and Tio (1990)	Axillary buds buds	1/2 RT 1 mg.l^{-1} 2,4 D	1 mg.l^{-1} 2,4 D Shoot	Callus
			0 mg.l^{-1} 2,4 D	Vitroplants
Hitmi et al. (1999)	Bud flowers	MS	4 mg.l^{-1} ANA + 0.4 mg.l^{-1} BAP	Callus
			2 mg.l^{-1} ANA +1 mg.l^{-1}BAP	Multiple shoots
			2 mg.l^{-1} ANA	Vitroplants

al., 1988; Zito and Tio, 1990). Because the pyrethrins accumulate in the flower heads, efforts to induce flowering *in vitro* might be expected to yield cultures with increased production of the pyrethrins. Staba and Zito (1985) and we (unpublished data) were unsuccessful in efforts to induce flowers in *in vitro* cultures, despite a range of conditions of temperature, light (photoperiods and light quality), and gibberellin addition.

B. Production of Pyrethrins

The rationale for phytoproduction (or production of products in plant cell culture) is that it ensures better control over supply, quality, and cost of raw materials in a manufacturing process, independently of social, political, economic, and climatic fluctuations.

1. Callus Cultures

Besides micropropagation, many authors have explored the potential production of pyrethrins by cultured cells. Pyrethrins have been reported in callus cultures of *Tagetes erecta* (Khanna et al., 1975; Cieniecka and Szybinska, 1992) and *Tagetes minuta* (Jain, 1977). The pyrethrins isolated from Jain tested positive for insecticidal activity. Chumsri and Staba (1975) reported that calli of *Chrysanthemum cinerariaefolium* and *Chrysanthemum coccinum* did not produce pyrethrins after gas chromatography and a mosquito larva bioassay analysis. Similarly, different workers have found that undifferentiated calli produce no detectable pyrethrins when analyzed by HPLC. However, this calli accumulate precursors (Cashyap et al., 1978; Zito and Tio, 1990). Kueh et al. (1985) identified chrysanthemic acid in undifferentiated calli and demonstrated by feeding ^{14}C-labeled chrysanthemic acid that glycoside rather than rethrolones esterify it. Zito and Tio (1990) analyzed calli, regenerated plantlets and leaves from 3-year-old greenhouse-grown plants for the two groups of pyrethrins, chrysanthemic and pyrethric acids and chrysanthemol alcohol (Table 3). They showed that the last part of the biosynthetic pathway is functional in the undifferentiated calli, but that they lack the ability to

TABLE 3
Relative Concentrations of Pyrethrins and Their Precursors (% dry wt) in *Chrysanthemum cinerariafolium* Leaves, Plantlets, and Callus

	Leaves	Plantlets	Callus
Chrysanthemyl alcohol	0.034 ± 0.007	0.017 ± 0.001	0.043 ± 0.002
Chrysanthemic acid	0.190 ± 0.150	0.867 ± 0.067	0.028 ± 0.001
Pyrethric acid	0.140 ± 0.019	0.905 ± 0.073	0.203 ± 0.012
Jasmolin I	0.019 ± 0.002	0.023 ± 0.002	Undetected
Cinerin I	0.017 ± 0.001	0.031 ± 0.004	Undetected
Pyrethrin I	0.535 ± 0.013	1.266 ± 0.087	Undetected
Jasmolin II	0.012 ± 0.001	0.094 ± 0.005	Undetected
Cinerin II	0.007 ± 0.001	0.092 ± 0.005	Undetected
Pyrethrin II	0.254 ± 0.003	0.825 ± 0.125	Undetected

esterify monoterpene acids in pyrethrins. They also found that the relative concentrations of chrysanthemic acid and pyrethric acid were about equal in leaves and plantlets. However, undifferentiated cells contained almost 10 times more pyrethric acid than chrysanthemic acid on a dry weight basis. Zito (1994) studied the content in pyrethrin and fatty acids and the fatty acid composition of *Chrysanthemum* cell cultures. Compared with flower heads, which contain 20.3%, the callus contained 8.4% and the plantlets 10.1% dry wt total fatty acids. Callus cultures lose the ability to produce nonadecanoic (19:0), heneicosanoic (21:1), pentacosanoic (25:0), octacosanoic (28:0), and tricontanoic (30:0) acids. The major fatty acids in the callus were palmitic (16:0), linoleic (18:2), and linolenic (18:3) acids. As the relative ratios of three major fatty acids found in leaves and regenerated plantlets are similar, while the callus produce a low amount of linolenic acid, this fatty acid occurs in the rethrolone biosynthesis, which explains the esterification of monoterpene acids by glucoside rather than rethrolones.

In 1976, a Japanese patent described the detection by gas chromatography and mosquito larva bioassay of pyrethrins in extracts of callus and redifferentiated plants (Aoki et al., 1976, 1978). The relationship between production rate of pyrethrins in parental clone, explant, and in cell cultures was analyzed (Zieg et al., 1983; Barthomeuf et al., 1996). Most of the initiated calli were able to synthesize and accumulate low pyrethrin content regardless of the explant origin. The authors concluded that there was a close correlation between pyrethrins content in *Chrysanthemum* callus cultures and that in original plants. The plant part used to initiate the cultures has little influence on pyrethrin production. Ravishankar et al. (1989) reported that maximal yield was reached at the end of the lag phase; however, in agreement with the results of Dhar and Pal (1993), we obtained a higher accumulation at the beginning of the stationary phase. These results suggest that the production of pyrethrins is associated with a period of slow growth. Rajasekaran et al. (1991) compared the bioefficiency of the pyrethrins from the two sources; pyrethrins extracted from 45-day-old callus tissue and standard extract from the Pyrethrum Board of Kenya. The comparative value of LC_{50} indicated that the toxicity to *Drosophila melanogaster* of the callus extract was almost 80% of that of standard pyrethrins. These authors suggested that further enhancement of the bioefficacy might be possible by the selection of cell lines that show higher levels of pyrethrins in cell cul-

tures. The repellency and knockdown properties of pyrethrins obtained from callus of *Chrysanthemum cinerariaefolium* and naturally derived standard pyrethrins were compared by Rajasekaran et al. (1996). With *Culex quinquefasciatus* (mosquito), the knockdown insects did not recover within the 24-h postexposure period, and standard extract was slightly superior in achieving 95% repellency. With *Tribolium castaneum* (beetle), there was no significant difference exerted by the extracts.

2. Organ Cultures

Generally, synthesis and accumulation of pyrethrins in the culture improved with the extent of cell differentiation, except for root cultures. It was found (Khanna et al., 1975; Jain, 1977) that roots differentiated from calli were unable to synthesize pyrethrins. Analysis of shoots that had differentiated from calli revealed the presence of the six esters (Zieg et al., 1983; Fujii and Shimizu, 1990). They concluded that the type of tissue organization had a bearing on the pyrethrin yields produced in *in vitro* cultures; they postulated that the formation of specialized structures such as surface oil glands and internal secretory canals is necessary for both pyrethrin synthesis and accumulation. Zito and Tio (1990) observed that the ratio of pyrethrins I to pyrethrins II in flower head and aseptic plantlet extracts was, respectively, 1.22:1 and 1.26:1, indicating that the accumulation of pyrethrins in the plantlets is similar to that in the flowers. The quality of the insecticidal activity of the pyrethrins has been compared in shoot cultures and in flowers; Cashyape et al. (1978) found that the relative proportions of the six esters, produced by shoot cultures from calli, was similar to that in 4-week-old seedlings. However, to compare the quality of the pyrethrins in shoot cultures and in natural flowers, the proportions reported by these authors are converted into percentage ratios (Table 4). The relative proportions of the six esters in the two types of tissue were not identical. The cinerins were far too low in shoots compared with flowers, while pyrethrins II were high. In extracts from our cultured cells (0.22% dry wt), a relative ratio of jasmoline II is about 80% (Hitmi, 1998).

3. Production Improvement

Various authors have studied the improvement of pyrethrin production by changes in environmental and culture conditions. Khanna and Khanna (1976) found that pyrethrin biosynthesis ability increased in callus cultures of *Tagetes erecta* when they added exogenous ascorbic acid to the medium. The influence of light on pyrethrin production was studied by Staba et al. (1984).

TABLE 4
Relative Amounts of Six Pyrethrins Produced by Differentiated Shoots and Flowers

Type of tissue	PyrI	CinI	JasmI	PyrII	CinII	JasmII
Shoots[a]	76.0	3.0	14.0	6.0	0.2	0.8
Ratio (%)[b]	81.7	3.2	15.1	85.7	2.9	11.4
Flowers						
Ratio mean (%)[b]	72.9	16.0	11.1	68.0	20.2	11.8
Ratio range (%)	65.3–84.2	4.5–27.2	7.2–15.8	53.1–79.2	7.9–390	7.3–16.2

[a] The six esters total 100%.
[b] Pyrethrins I and II each total 100%.

They observed that light significantly enhanced the production and/or accumulation of pyrethrins in shoot cultures that received 4000 lux for a 16-h photoperiod. Our work showed that biomass yield and pyrethrin content in cell cultures were higher when they were grown in the light than in the dark. The optimal illumination was found to be 60 $\mu mol.m^{-2}.s^{-1}$ (Hitmi et al., 1998). This may be due to the reduced levels of endogenous monoterpene-degrading enzymes in the light, such as epoxidase, and the formation of toluene and acetone by cells at the expense of monoterpenes during dark cultures (Charlwood et al., 1989). We also observed that cells cultured under red or blue light at 60 $\mu mol.m^{-2}.s^{-1}$ had reduced biosynthetic ability. The medium composition also influence growth and pyrethrin production in leaf calli of *Chrysanthemum cinerariaefolium* (Rajasekaran et al., 1990); it has been found that relative proportions of carbon, nitrogen, and phosphate of 2:1:2 were necessary for optimal growth of calli. Rajasekaran et al. (1991) found that nitrogen stress-enhanced production. Although nitrogen is not a constituent of pyrethrins, its depletion affects primary metabolism and growth, thereby channeling the available primary metabolites (remote precursors) toward pyrethrin biosynthesis. Conversely, sugar or phosphate stress impaired synthetic ability, indicating that pyrethrin biogenesis has a relatively high energy requirement, and hence its biosynthesis may be affected by lowered ATP metabolism as a result of phosphate depletion and reduced glycolysis due to sugar depletion. They concluded that in the future nitrogen-stress medium could find an application in the scaling up of cell cultures for pyrethrin production using a two-stage culture method. In addition, we have demonstrated that MS media is better than other basal media (Gamborg, White, Heller). MS medium diluted twice induced a higher biosynthetic ability, whereas dilution to 1/3 induced a decrease in biomass yield and pyrethrin production (Hitmi et al., 1998). The influence of formaldehyde on growth and pyrethrin production in *in vitro* cultures and tissues of *Chrysanthemum cinerariaefolium* has also been studied (Nirmala et al., 1992). It was observed that formaldehyde concentrations of up to 0.025% slightly inhibited callus growth, but formaldehyde addition in the range of concentrations 0.05 to 0.2% enhanced pyrethrin production. In a study of growth and pyrethrin formation by selected cell lines of *Chrysanthemum cinerariaefolium,* Pal and Dhar (1985) found that the production of pyrethrins in callus cultures was influenced by the nature and concentration of growth regulators (auxins and cytokinins). They established that BA (benzyl adenine) induced better growth than other cytokinins, and that NAA inhibited production. They also reported that the 2,4-D (2,4-dichlorophenoxyacetic acid) induced optimal growth and pyrethrin synthesis, and they obtained the highest production of pyrethrins in the callus lines tested with 0.5 $mg.l^{-1}$ of 2,4-D and 0.5 $mg.l^{-1}$ of BA (Dhar and Pal, 1993). We have selected a *Chrysanthemum cinerariaefolium* cell line that produce pyrethrins at reduced. 0.46% dry wt (unpublished data).

4. Cryopreservation of Selected Cell and Tissues Lines

Usually, when cultures are maintained through several subcultures, the yield of interesting compounds decreases or become nil (Seitz, 1987). For pyrethrins such a decrease in synthetic capacity with subcultures has been observed (Sarker and Pal, 1991). We have developed a method for cryopreserving highly productive cell lines in which the thawed cells are able to regrow and produce pyrethrins after cryogenic storage. This technique comprises three phases:

a pregrowth period where the calli are cultured in half-strength MS nutrient medium containing 0.5 *M* sucrose for 30 days, then incubated in the same medium in the presence of 5% DMSO for 1 h in an ice bath, cooled at 1°C.min^{-1} to –20°C, stored for 24 h at this temperature, and immersed in liquid nitrogen. After cryopreservation, the cells conserved the same growth pattern but displayed different biochemical properties. A high regrowth rate was obtained after this treatment (96.2%). The subculture derived from the thawed cells was characterized by a higher pyrethrin production (0.18%) and pyrethrins I to II ratio (1.31) with respect to control of 0.16% and 1.19 (Hitmi et al., 1997). We used the same cryopreservation protocol to obtain the regrowth of *Chrysanthemum cinerariaefolium* shoot tips of *in vitro* plantlets. The pretreatment comprise a preculture of explanted stem apices for 3 days with 0.55 *M* sucrose and incubation for 1 h in medium with 7.5% DMSO. The morphogenic potential of frozen stem apices and the total chlorophyll and pyrethrin contents of regenerated plants were preserved after storage in nitrogen liquid (Hitmi et al., 1999).

5. Cell Suspension Cultures

Whereas much attention has been directed to callus and organized tissues, little work has been done on suspension cultures. Bretler and Tramper (1985) failed to obtain a stable cell suspension from *Chrysanthemum cinerariaefolium* calli, but they succeeded with *Chrysanthemum coccinum* calli. A low density characterized these suspensions, a doubling time of 1 day and a production of 7 g.l^{-1} dry cells. Unfortunately, these cultures proved unable to accumulate pyrethrins. Cell suspension cultures able to produce pyrethrins were obtained by Cieniecka et al. (1993a). They increase the synthesis capacity of cells by adding fungal elicitors (Cieniecka et al., 1993a) and ascorbic acid (Cieniecka et al., 1993b) to the liquid culture medium. Jovetic and De Gooijer (1995) reported that in the early 1980s, the McLaughlin Gormley King Company (Minneapolis) tried to develop an *in vitro* culture process for the production of pyrethrins. They failed to establish a stable productive suspension culture. For the production of pyrethrins they concluded that a partial differentiation in tissue cultures is compulsory. They developed a prototype bioreactor for the culture of differentiated tissues. However, the project was discontinued after several years of development work, because large-scale plant shoot culture was not yet feasible. The design of bioreactors with new configurations is crucial to cultivate highly differentiated tissues on an industrial scale (Whitney, 1992). Scaling-up of shoot cultures and productivity improvements are the main problems associated with designing a bioreactor suitable for an industrial process.

6. Bioconversion

The use of plant enzymes or genetically engineered microorganisms for bioconversion of pyrethrin precursors might afford an alternative to conventional pyrethrin production.

McLaughlin Gromley Company (Minnesota) patented an enzymatic synthesis of pyrethrins in 1984. The production process comprised a preparation of cell-free homogenate containing enzymes and cofactors necessary for the pyrethrin synthesis pathway of *Chrysanthemum cinerariaefolium* and *Tagetes* spp., and an incubation of the homogenate with radiolabeled mevalonic acid or isopentenyl pyrophosphate. In addition to pyrethrin production, this process can be used to produce chrysanthemyl alcohol by hydrolyzing chrysanthemyl alcohol phosphate.

For genetic engineering, it is necessary to identify and characterize the enzyme(s) catalyzing the pyrethrin synthesis and the gene(s) responsible for the synthesis of these enzyme(s). It is interesting to note that different stages of the pyrethrin biosynthesis pathway have been observed in the biochemical machinery of other plants and microorganisms. Chrysanthemyl alcohol can be oxidized by *Aspergillus ochraceus* into chrysanthemic acid (Rajasekaran et al., 1991; Nirmala et al., 1992), and cinerone is converted into cinerolone by *Aspergillus niger* (LeMathieu et al., 1968; Davis and Miski, 1988). The fungus *Botryo diplodra* is able to produce pyrethrolone (Miersch et al., 1989). These findings interested the American company, AgriDyne Technologies Inc. (Utah), which decided to use genetic engineering to develop a precursor of pyrethrins (also important in the chemical production of synthetic pyrethroids) (Shand, 1992). They isolated the gene coding for chrysanthemyl diphosphate synthase and are testing its activity and production levels in microorganisms (Jovetic and De Gooijer, 1995). In parallel, The Advanced Technology Program (ATP) of the National Institute of Standards and Technology (NIST) planned production of the pyrethrin precursors using recombinant yeasts (Perrier, 1997). Chrysanthemyl diphosphate synthase, the only enzyme to be expressed in a microorganism, is just one of the enzymes acting in the biosynthesis of pyrethrins. Because pyrethrins are a mixture of six esters the number of genes involved in their biosynthesis is unknown, even though the mechanism involved is polygenic inheritance. Another problem is related to insufficient knowledge of the metabolic step involved in the monoterpene ester biosynthesis (Bhat, 1995).

An industrial production of pyrethrins based on bioconversion of readily available precursors may be much more attractive for industrial development than the process based on plant cell/tissue cultures. This is owing to uncertainty concerning the expression of the enzymatic activity of cell-free homogenate and the enzymes in microorganisms.

To our knowledge, no hemisynthetic mode of production has been published, possibly because:

1. This production route is economically nonviable
2. The compounds obtained offer no advantage over the pyrethrinoids
3. Research is still in the development stage

7. Biosynthetic Aspects in Cell and Tissue Cultures

Because of the highly seasonal supply of *Chrysanthemum cinerariaefolium* flowers, tissue cultures that produce adequate quantities of pyrethrins would be more convenient for biosynthetic study. After the success of cell-free homogenate preparation from flower heads (Staba and Zito, 1985), *in vitro* tissues were investigated for their ability to synthesize pyrethrins and their precursors by incubation with [1-^{14}C]-isopentenylpyrophosphate ([1-^{14}C]-IPP). They found that in shoot cultures, the incorporation of [1-^{14}C]-IPP into pyrethrins I was 0.9%. They also showed that the cold carrier addition of pyrethrins to callus-free homogenate incubated with [1-^{14}C]-IPP prevented the uptake of radioactivity by the pyrethrins. However, Zito et al. (1991) have established that the cell-free homogenate from callus cultures is an excellent source for the study of the enzymes involved in the formation of the pyrethrin monoterpene precursors, that is, chrysanthemyl alcohol, chrysanthemic acid, and pyrethric acid. They studied the activity of isopentenyl-pyrophosphate isomerase (EC 5.3.3.2; isopentenyldiphosphate delta 3-delta 2-isomerase), prenyltransferase activity (EC

25.1.11; allylic-terpene-diphosphate: isopentenyl diphosphate terpenoid alyltransferase), and alkaline phosphatase (EC 3.1.3.1.; orthophosphoric-monoester phosphohydrolase (alkaline optimum). They determined their specific activity, respectively, as $6.9 \pm 0.01\ 10^5$ dpm.mg^{-1} protein/min, $0.44 \pm 0.03\ 10^5$ dpm.mg^{-1} protein/min and a negligible value. All these enzyme activities were also found to require divalent metal ions, with 2 m*M* Mg^{2+} being optimal. They were active over a pH range of 6.8 to 7.5, with 7.5 being optimal. Iodoacetamide up to 1.10^{-3} inhibited none of the enzyme activities, although iodoacetamide is known to inhibit isomerase and prenyltransferase from other sources (Crombie, 1995).

IV. ECONOMIC ASPECTS

The ultimate criterion for commercializing a plant cell culture process for the production of secondary metabolites is economic. It has to be less expensive to make a product by *in vitro* culture than to extract it from traditional agricultural material or to produce it by chemical synthesis.

A. General Aspects

For the successful application of a plant tissue culture concept to the process industries, the essential criteria of product quality and product cost must be met. Several cost-price calculations to produce secondary metabolites have been published (Sahai and Knuth, 1985; Verpoorte et al., 1991). Verpoorte et al. (1993) compared the actual cost of some secondary metabolites and demonstrated that for only a few products could the market price warrant a plant biotechnological production. Sahai and Knuth (1985) compared the *in vitro* production for a hypothetical plant product at different concentrations at an annual production capacity of 20,000 kg. An increase in product concentration from 0.1% to 1% dry wt results in a 16-fold reduction in fixed capital investment and a lowering of total production cost. A similar pattern was found by Verpoorte et al. (1993) using ajmalicine (an alkaloid extracted from *Catharanthus roseus*) as a model. They also compared the effect of the production volume and found that a profitable venture is viable at production capacities ranging from 730 kg/year to 3650 kg/year. Unsurprisingly therefore, shikonin, the first plant tissue culture product to be commercialized (Mitsui Chemicals Japan), is a high value product ($4500/kg). They concluded that industrial production by biotechnology based on cell culture is capital-intensive, requiring a high initial capital investment and relying heavily on sophisticated technology, and for intermediate or low production capacities the product value should be greater than $500/kg. Other workers (Balandrin and Klocke, 1988) have estimated that production of secondary metabolites in plant cell cultures is economically attractive only for cases producing more than 1 g of compound per litter of cell culture and for compounds with a value exceeding $500 to $1000 per kg. The plant secondary metabolites produced industrially by biotechnology are cited in Table 5 (Ushiyama, 1991; Petersen and Alfermann, 1993).

B. Implications for Pyrethrins

Despite the lack of data on product yields and volumetric productivity in the industrial production of pyrethrins by biotechnological processes, some estimation can be derived from the available information. For these estimations we considered market demands, price fluctuations, and dumping, alternative supply sources, and socio-political aspects. Payne et al. (1992) suggested a

TABLE 5
Secondary Metabolites Produced Industrially by Plant Cell Cultures

Product	Species	Company
Berberine	*Coptis japonica*	Unknown
Ginsenosides	*Panax ginseng*	Mitsui Petrochemical Ind. Ltd.
Purpurin	*Rubia akane*	Nitto Denko Corp.
Shikonin	*Lithospermum erythrorhizon*	Mitsui Petrochemical Ind. Ltd.

market share of $10 million per year as a threshold for the commercialization of any plant tissue process. According to the French company Francereco, production by plant tissue culture could be justified only for rare products that are costly and difficult to obtain by other means. Specifically, they estimated that this approach would be feasible only for products whose world annual potential market would be $20 to $50 million, with a minimum selling price of about $500/kg (Sasson, 1993). The global market of pyrethrins was estimated at $400 million annually. Hence, if 5 to 10% of the world market share were captured by biotechnological processes, which is a realistic estimate, annual sales could reach 20 to 40 million dollars; therefore, the production of pyrethrins by plant tissue cultures can be regarded as economically attractive (Jovetic and De Gooijer, 1995). Verpoorte et al. (1993) estimated that biotechnology production of pyrethrins is not worthwhile industrially due to the low yields cited in the literature. We have previously selected a stable *Chrysanthemum* cell line able to produce 0.46% dry wt, higher yields have already been obtained and our aim is to improve the yield to 1% dry wt, which make pyrethrin production economically viable by this route.

For bioconversion, AgriDyne Technologies, Inc., hope to produce pyrethrins within an estimated price range of $110 to $150/kg and sales could reach $100 million by the late 1990s (Jovetic and De Gooijer, 1995). However, the technological feasibility of this approach is still uncertain, and levels of production and enzyme activities in culture will ultimately determine the economic viability.

CONCLUSION

The pyrethrins, the most economically important natural insecticides, have been used for about 150 years and have survived frequent challenges to their economic relevance. The use of plant cell cultures for the biotechnological production of pyrethrins has a number of advantages. Accordingly, the development of efficient biotechnological processes able to improve the production of pyrethrins and their use as an alternative to the traditional extraction procedures is a current challenge. Pyrethrin synthesis and accumulation in culture is determined by the extent of cell differentiation. Numerous researchers have reported that undifferentiated calli or calli differentiated in roots do not produce pyrethrins, whereas cultures showing high differentiation, such as shoot cultures, invariably produced pyrethrins in large amounts. However, it is now well known that high-yield callus cell lines produce pyrethrins and could be used as an alternative raw material for the extraction of pyrethrins. To our knowledge, the best result obtained with callus culture was about 0.5% and was obtained in our laboratory. New results obtained shortly indicated that the yield could be improved to about 1%. Cell suspension cultures were unable to accumulate pyre-

thrins and little information is available on their growth stability over time. To prevent this reduction or loss of ability to synthesize the secondary metabolites, high-producing cell lines cryoconservation methods were developed. Industrial processes with callus and suspension cultures have not appeared economically viable. Even so, large-scale culturing of plant cells is in general technologically feasible. Shoots produce significant amounts of pyrethrins and could be potential candidates for an industrial process. There will be advantages to using organized cultures rather than undifferentiated cell cultures, which often tend to show aberrant expression of secondary pathways and tend to be biochemically unstable. Given our present knowledge and the state of technology, plant cell/tissue cultures do not look promising for industrial production of pyrethrins. The common view of authors is that a number of problems have to be solved before pyrethrins can be commercially produced by plant cell/tissue cultures. Studies in the development of biotechnological processes may help improve our knowledge of pyrethrins biosynthetic mechanisms. As undifferentiated calli are able to produce the irregular monoterpene pyrethrin precursors, they can be used to elucidate biosynthetic reactions leading to pyrethrins. Today, the identification of biochemical and/or genetic markers for secondary metabolites production is regarded as essential. Indeed, the identification of the specific key enzymes in the biosynthetic pathway of pyrethrins is obviously a prerequisite for any bioconversion or genetic engineering programs.

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