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# PRODUCTION OF SECONDARY METABOLITES IN PLANT CELL CULTURES

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## INTRODUCTION

Since man's early days, plants have been the source of drugs, cosmetics, agrochemicals, flavours, perfumes, sweeteners, pigments and enzymes. The secondary metabolites occurring naturally in intact plants and of intense commercial interest include pharmaceuticals, fine chemicals and industrially useful biochemicals, Table 1.

Table 1: Major groups of compounds with commercial importance derived from plants.

Chemical Class	Compound	Plant Species	Industrial Uses
Alkaloid	Codeine	<i>Papaver somniferum</i>	Analgesic
	Lobeline	<i>Lobelia inflata</i>	Respiratory stimulant
	Quinine	<i>Cinchona ledgeriana</i>	Antimalarial, Bittering agent
	Atropine	<i>Atropa belladonna</i>	Pupil dilation and ophthalmic practise
	Hyoscyamine	<i>Datura stramonium</i>	Anticholinergic
	Scopolamine	<i>Datura stramonium</i> <i>Duboisia myoporoides</i>	Antihypertensive, Treatment of motion sickness
	Vincristine, Vinblastine	<i>Catharanthus roseus</i>	Antileukaemic
Terpenoid	Diosgenin	<i>Dioscorea deltoidea</i>	Antifertility agents
	Digoxin	<i>Digitalis lanata</i>	Cardiotonic
	Saponins	<i>Panax ginseng</i>	Tonic
	Isoprenoids	<i>Hevea brasiliensis</i>	Rubber
	Jasmine	<i>Jasminum</i> sp.	Perfume
	Pyrethrin	<i>Chrysanthemum cinerariaefolium</i>	Insecticide
Phenolics	Betacyanin, Betaxanthin	<i>Beta vulgaris</i>	Pigment
	Shikonin	<i>Lithospermum erythrorhizon</i>	Anti-inflammatory pigment
	Thaumatococin	<i>Thaumatococcus daniellii</i>	Non-nutritive sweetener
Polyssacharides	Gum arabic	<i>Acacia</i> sp.	Gums
Proteins	Papain	<i>Carica papaya</i>	Proteases



Supply of natural plant products is often reduced by the effect of environmental stress (climatic and seasonal variation) and other depredations on plant yields (pests and diseases), by the long life cycle of some plants and by the frequent need of large amounts of plant material. On the other hand, these compounds are isolated from the plants in more or less complex extracts, thus lowering the yield due to successive purification steps.

The same active compound can be obtained from different plant species. The choice is done having in mind, among others, the yield of the extraction, the chemical nature of the compounds, the cost of the plant, the cost of maintenance of the product and the absence of harmful components. So it seems that the major problem related to the obtention of natural plant products is the rentabilization of the production of the active compounds, which must be done through a selection of best varieties, and a guarantee of product homogeneity and quality.

Several alternative procedures have been developed to overcome these problems: 1) total chemical synthesis (when the molecules are not too complex and as such the needed reactions are not too expensive) or semisynthesis and 2) plant *in vitro* culture, not only to obtain better varieties but also as a source of primary and secondary metabolites, similar or not to those produced by the mother plant. When new types of compounds are produced they can also be used as raw material in the synthesis of new chemicals.

The main goal of the industries is to obtain, sooner than by the traditional methods, new products, varieties resistant to diseases, or increased production of compounds. In this field, plant cell cultures offer additional advantages: 1) they are independent of environmental conditions, such as climate, pests, geographic and seasonal variations, 2) they are a defined production system, with low space necessities, allowing the satisfaction of market needs, 3) they are an easily working system, since it is possible to have them under nutritional (culture medium composition, pH) and environmental (light, temperature, aeration, shaking) control, and to change the parameters under study, 4) They allow, in some cases, the obtention of a high production yield and 5) they broaden the perspectives on the obtention of new products.

## POTENTIALITIES OF PLANT CELL CULTURES

Although plant cell cultures are potential sources of valuable pharmaceuticals and other biologically active phytochemicals, only relatively few cultures synthesize secondary compounds, over extended periods, in amounts comparable to those found in the whole plant. Frequently, no secondary metabolites characteristic of the intact plant are produced, and the costs associated with the cultures maintenance have not allowed profitable commercialization of plant cell secondary metabolite. A notable exception is the production of shikonin by *Lithospermum erythrorhizon* cell cultures. Apart from this there are at least more than 20 examples in which plant cell cultures, either *calli* or cell suspensions, have been shown to produce yields equal to, or higher than the mother plant.

Due to several drawbacks (slow growth, cell aggregation, shear stress sensitivity, diffusion limitations, formation of secondary metabolites after growth has ceased, which implies increased

fermentation growth time) most of the processes involving plant cell cultures are only economically viable when the synthesized compounds are costly plant specific [5]. Despite these limitations, plant cell cultures are still a real alternative way in several fields:

### MICROPROPAGATION

Micropropagation shows many potentialities in the multiplication and maintenance of interesting plant species that require several years of development. From the medicinal point of view, the micropropagation of plants such as *Cephaelis ipecacuanha*, *Panax ginseng*, *Digitalis lanata*, *Camomilla recutita* and *Papaver somniferum* are promising examples [17]. Also in the field of essential oil industry several species are currently micropropagated to attend to market needs: *Pelargonium graveolens*, *Mentha arvensis*, *Syzigium aromaticum*, *Pimenta dioica*, *Vanilla planifolia*, *Rosa* sp. and *Curcuma longa* [45]. Somaclonal variation has also been employed as a means to select plants with altered levels of phytochemicals with medicinal and industrial interest. The major handicap of this procedure is the lack of stability in the obtained chemovariants [17].

### TRANSGENIC PLANTS

The best known models of genetic modifications of plants are related to the enhancement of plant performance (herbicide, insect resistance) and of productivity (pigmentation, storage protein) [17]. Modification of *Brassica* plants, to produce oil of specific chemical composition, has been the objective of several laboratories [17].

### HAIRY ROOTS

Hairy roots are autonomous roots obtained by transformation with *Agrobacterium rhizogenes*. A massive increase of biomass over relatively short culture periods and complete differentiation of root tissue warrant the production of root specific phytochemicals at substantial levels [17, 33]. As an example, hairy root cultures of *Papaver somniferum* produced sanguinarine at levels up to 2 % of dry weight [17]. Furthermore, productivity as well as chromosome number appears to be more stable in these cultures than in the normal ones [33].

### PRODUCTION OF SECONDARY METABOLITES

*In vitro* production of higher levels of plant specific products or of products from plants whose traditional culture is not easy, superior to those produced by the nature plants, has been the aim of



several studies [27]. There are several examples of high - producing cell suspension cultures giving rise to anthraquinones, peptides, alkaloids and putrescines, among others. Cultures of *Morinda citrifolia* produce alizarin with a yield of 18 % dry weight. Similar yields of rosmarinic acid were obtained in cell cultures of *Coleus blumei*. The cultures of *Syringa vulgaris* produce a polyphenolic glycoside with a yield of 16 % dry weight [5]. Other examples exist of plant cell cultures that, producing compounds of high pharmaceutical interest, have low yields, which could be improved by adequate selection procedures [5]. Among these we can refer to the cultures of *Panax ginseng*, *Glycyrrhiza glabra*, *Solanum* sp., *Ammi visnaga*, *Cassia tora*, *Lithospermum erythrorhizon*, *Macleaya microcarpa*, *Datura* sp., *Scopolia parviflora*, *Coffea arabica*, *Papaver* sp., *Ephedra gerardiana*, *Coptis japonica* and *Cephalotaxus harringtonia* [5].

Owing to the conditions in which the plant cultures are maintained some new compounds, never detected in the mother plant, have been produced. The examples of new compounds include various classes of sesquiterpenes, anthraquinones, furanocoumarines and alkaloids [30]. The most remarkable feature of the essential oil isolated from cell cultures of *Achillea millefolium* was the presence of demethoxyencecalin, a chromene with a good insecticide activity against several insect species, that belongs to the benzopyran group. This group of compounds have never been isolated from the Tribe Anthemideae to which *A. millefolium* belongs [29].

#### BIOTRANSFORMATION

Biotransformation of low cost exogenously applied precursors, when the products are not efficiently produced by chemical or microbial means, is a technique to explore [5, 47]. Some of the plant characteristic reactions, such as position - specific glycosylation of a multifunctional substrate appear to have interesting potential for biotechnology, since, in many cases, such glycosides either cannot be synthesized by chemical means or only with the tedious introduction of protective groups. Biotechnological application of this potential does, however, require rigorous selection of suitable cell strains because, in most cases, plant cells have shown to carry out more than one biotransformation reaction with a given substrate. An example of success in this field, is the one of the cell suspension cultures of *Stevia rebaudiana* which convert steviol in stevioside, compound widely used as a natural sweetening agent [5]. One of the main advantages of biotransformation is related to the fact that most of the products formed are excreted into the culture medium. With the advances made in the last years, some of the processes have reached a level where they can be considered for commercial application.

#### IMMOBILIZATION

Immobilized plant cells appear as an alternative to the usual techniques of secondary metabolites plant cell culture production. This system allows: 1) easy separation of the cells from the

culture medium and, as such, the reuse of the biomass and the extraction of the products from the medium and 2) more efficient continuous use of fermentors because of their high biomass / volume. The different sensitivity of plant cell cultures to shear, osmotic and nutritional stresses, temperature, pH, oxygen and chemicals, coupled with the formation of cell aggregates, determines the method of immobilization. Indeed, the use of immobilized plant cell cultures seemed very promising in procedures that dealt with synthetic or degradative reactions involving expensive cofactors or complex metabolic pathways. Nevertheless, several limitations were evident, such as difficulties in membrane substrate, or product, permeabilization, side reactions due to other enzymes present in the cells, or even to toxic substances released through degenerative cells. On the other hand, secondary metabolites were frequently entrapped by the immobilization matrix. In a tentative to overcome this problem several authors have assayed permeabilization procedures, however without promising results [9].

### METABOLIC PATHWAYS

The presence of chlorophylls and phenols in the extracts of in nature grown plants is, most of the times, a drawback in the metabolic pathways study. Plant cell cultures are, in this case, an alternative material to study primary or secondary metabolic pathways [37]. In cultures of *Catharanthus roseus*, it was possible to isolate and determine, for the first time, the structure of several compounds as well as the enzymes of the metabolic pathway of ajmalicine, that were not easily detected using *in vivo* plant material [5]. Likewise, the studies on the biosynthesis of paniculides (sesquiterpenes), in cultures of *Andrographis* and on the formation of triterpenes in cultures of *Isodon japonicus* have allowed the elucidation of some steps of the biosynthesis of terpenes [3, 5].

### SECONDARY METABOLITES YIELD ENHANCEMENT

Several factors have been reported to improve the *in vitro* synthesis of plant products. These include culture conditions (differentiation, nutrition, hormone levels, irradiation, temperature, pH, aeration), two phase system cultures, precursor feeding, immobilization, hairy roots and elicitation.

### DIFFERENTIATION

*Calli* cultures have been used by several authors to study the *in vitro* production of secondary metabolites [7, 13, 16, 19, 31, 48]. This type of cultures has been chosen because the solid media on which the tissues are maintained simulates nutritional gradients as those observed *in vivo* [2, 3, 16]. The results obtained are highly variable, species dependent and, most of all, they depend on the differentiation of the cultures [7, 13, 16, 19, 31, 48]. For instance, numerous reports support the view



that there is a correlation between essential oil production and cell differentiation [3, 12, 13, 16, 40]. Nevertheless, tissue differentiation is not always determinant in *in vitro* essential oil production, which may indicate that, at least in some conditions, the synthesis is possible in less organized systems [13, 20, 21]. *Calli* cultures of *Achillea millefolium* ssp. *millefolium* (yarrow) maintained in Gamborg B5 medium, supplemented with  $1.5 \text{ mg.l}^{-1}$  2,4-D/ $0.1 \text{ mg.l}^{-1}$  Kin have not shown increased yield of essential oil when compared to the cell suspension cultures grown in the same medium [29].

## MEDIUM COMPOSITION

*In vitro* production of secondary metabolites is affected by culture medium composition and is plant species dependent [7, 30, 36, 43].

The analyses on the production of essential oil by cell cultures of *Thymus vulgaris* have shown that, from the five culture media tested, MS medium was the one that most induced terpene production [48].

In cell suspension cultures of *Ononis natrix* maintained in MS medium supplemented with NAA / Kin [35], the accumulation of medicarpin and maackiain was sugar dependent. From the three carbon sources assayed (sucrose, glucose and fructose), the higher accumulation of both pterocarpanes was attained in medium containing sucrose (Miguel and Barroso, unpublished results). Other authors have also reported lower yield of rosmarinic acid in plant cell cultures maintained in medium supplemented with glucose [24].

Nitrogen concentration has been found to affect the level of several products in cell suspension cultures [30, 41, 42, 50]. The accumulation of medicarpin and maackiain was higher in cell cultures of *O. natrix* grown in MS medium containing 4 g  $\text{KNO}_3$  as sole nitrogen source (Miguel and Barroso, unpublished results).

Hormone composition is another critical factor in secondary product accumulation. Sugisawa *et al.* [48] have shown that from the different combinations of growth regulators assayed the supplementation with 2,4-D (10 ppm) / Kin (10 ppm) increased five times the essential oil production. For cell suspension cultures of *O. natrix* grown in medium supplemented with 2,4-D / Kin the accumulation of medicarpin and maackiain was lower than that attained in medium supplemented with NAA / Kin (Miguel and Barroso, unpublished results). Likewise, Ozeki *et al.* [38, 39], reported the inhibition of anthocyanins synthesis in carrot suspension cultures grown in medium containing 2,4-D. These results seem to be due to an inhibition of the synthesis of mRNAs coding *l*-phenylalanine ammonia lyase (PAL) and chalcone synthase, two key-enzymes of isoflavonoids metabolic pathway.

## PHYSICAL CONDITIONS

Light, temperature, medium pH and aeration have shown to affect secondary product accumulation in several cell cultures.

According to Corduan and Reinhard [18] the biosynthesis of essential oils in *Ruta graveolens* was shown to be dependent on the quantity and quality of the light. The essential oil composition of cultures grown under continuous light (Cool White, 250 lux) was similar to that of the mother plant, while the cultures grown in darkness or under other wavelengths produced an essential oil with different composition. Nevertheless, in several systems, the production of essential oil by cell cultures does not seem to be dependent on the presence of light [36]. Figueiredo [29] observed an increase in essential oil yield when the cultures of *Achillea millefolium* were transferred from photoperiod to darkness conditions.

In general, secondary metabolites accumulation in culture is not enhanced by temperatures above 25 °C. Cultures grown at 30 °C quickly brownish and die [36, 49].

Although less explored, as a mean to improve essential oil production in culture, the addition of CO<sub>2</sub> has shown to stimulate the synthesis of monoterpenes in cultures of *Vitis vinifera* and of the aroma constituents of cultures of *Malus* sp. [36]. Likewise, according several authors, agitation and O<sub>2</sub> tensions had significant effects upon the growth and the accumulation of secondary metabolites in cell cultures [30, 32, 44].

#### PRECURSORS FEEDING

The addition of precursors from the biosynthetic pathways of terpenes, to the culture media, has been referred to stimulate the synthesis of essential oil [2, 3, 36].

The addition of organic acids to *calli* cultures of *Pelargonium fragans*, led to a 225 fold increase in terpene production, with a coupled reduction of cell growth [13]. On the other hand, the addition of mevalonic acid to the culture medium of *calli* of *Thymus vulgaris* led to a 12 fold increase in the production of essential oils [48]. It is noteworthy, however, that the effect of secondary product precursors in plant cell cultures is diverse and very often contradictory [22].

#### IMMOBILIZATION

Immobilized cell systems have been used to induce higher accumulation of secondary metabolites in plant cell cultures. The increased yield seems to be due to the higher cellular aggregation. With immobilized cells of *Pelargonium fragans*, the yield of the essential oil increased 8 fold relatively to the non-immobilized ones. For all immobilized cultures, more than 90 % of the oil accumulated in the culture medium [12, 13].

#### CULTURE IN TWO PHASE SYSTEM

In an attempt to avoid the toxicity of some compounds excreted into the culture medium, namely some components of the essential oils, several authors have been using the so called two



phase system [10, 13, 19, 51]. This system is based on the addition of an adsorbent to the culture medium that temporarily accumulates the products excreted by the cells.

The addition of a non-polar adsorbent (Lichroprep RP-8) allowed the accumulation of low amounts of monoterpenes in cultures of *Mentha piperita* [19] and of anethol in cultures of *Pimpinella anisum* [36]. In several cases the addition of Miglyol (a non-toxic triglyceride water immiscible) to the culture medium, was suitable for recovering the essential oils produced by plant cell cultures. This method revealed useful, not only for the quantitation of the excreted terpenes, but also for the detection of terpenes that, by their volatility, are not usually isolated from cell cultures [8, 10, 13, 36]. The higher amounts of products recovered using this system seems to be due to their greater accumulation in Miglyol rather than a *de novo* synthesis. According to Cresswell *et al* [22], the increase of the product yield results from the protection of the volatiles that would be otherwise subject to either the aqueous and oxidative conditions occurring *in vitro*, to the reduction of feedback mechanisms that inhibit the production of primary or secondary precursors, or to the derepression of the genome.

The profile of the compounds excreted into the culture medium can also be affected by the addition of this triglyceride [13]. Depending on the species under study, the presence of Miglyol in the culture medium may, or may not, affect the cell growth [8, 10, 13, 22]. Of the different methods assayed, in order to improve the yield of the essential oil produced by cell cultures of *Achillea millefolium*, those grown in B5 medium with Miglyol showed a higher yield (0.002 %, w/w) and a wider range of compounds [29].

## ELICITATION

Many plants respond to microbial or fungal attack by producing low molecular weight compounds called phytoalexins [34]. These compounds accumulate rapidly and at high concentration at the site of infection and in the surrounding tissues, preventing the growth of the microorganism [23, 34].

The molecules that trigger the accumulation of phytoalexins are called elicitors. This term refers to both biotic (complex carbohydrates, proteins, glycoproteins, unsaturated fatty acids) and abiotic elicitors (UV radiation, heat, cold, ethylene, fungicides, antibiotics, heavy metals, high salts concentration) [25, 26]. Regarding biotic elicitors, they comprise both plant-derived (endogenous elicitors) and microorganism-derived elicitors [1, 6, 23, 34].

Elicitation has recently been explored in order to improve the accumulation of secondary metabolites in plant cell cultures and, in several cases, has given very good results. This technique shows as main features: 1) short culture periods following induction, 2) it does not require transfer of cells to a new production medium, 3) metabolites are generally excreted into the culture medium, 4) the cells can be re-elicited, leading to a semicontinuous production process, 5) elicitor-treated cells can be used in biotransformation assays and 6) new compounds can be obtained following elicitation [14, 17, 26].

Stimulation of secondary metabolism in elicitor-treated cells is affected by several factors, some of which are linked to the elicitor and others to the cultured cells.

### ***Elicitor specificity and elicitor concentration***

Secondary metabolism of cell suspension cultures can be triggered by the addition of complex elicitor preparations obtained from either virus, bacteria or fungi. These crude preparations are usually used since the attempts to characterize the elicitor compound of such mixtures have revealed the presence of more than one active component. Such homogenates showed, in several cases, higher activity than the isolated active compounds due to an additive or synergistic effect of the elicitors [26]. Several examples of elicitor preparations are provided in Table 2.

Table 2: Elicitor preparations used to induce accumulation of secondary metabolites in cell cultures.

Elicitor	Preparation
<i>Pythium aphanidermatum</i>	Filtrates, extracts and homogenates
<i>Eurotium rubrum</i>	Filtrates and extracts
<i>Micromucor isabellina</i>	Filtrates and extracts
<i>Chrysosporium palmarum</i>	Filtrates and extracts
<i>Botrytis</i> sp.	Homogenates
<i>Dendryphion</i> sp.	Extract
<i>Aspergillus niger</i>	Homogenate
<i>Candida albicans</i>	Autoclaved cells
<i>Phytophthora cinnamomi</i>	Autoclaved mycelium
Yeast	Carbohydrate preparation
Nigeran	-

It should be said, however, that the elicitation does not always work. This raises the question on the specificity of the elicitor. Homogenates of *Botrytis* sp. promoted the accumulation of isoflavan in cell cultures of *Phaseolus vulgaris* and sanguinarine in *Papaver* cultures but did not trigger the accumulation of indole alkaloids in cultures of *Catharanthus roseus* [26]. Cell suspension cultures of *Hyoscyamus albus*, treated with *Candida albicans* autoclaved cells or freeze dried *Phytophthora cinnamomi* autoclaved mycelium, accumulated both lubimin, 10-*epi*-lubimin, rishitin and solavetivone, in contrast to those treated with cellulase that did not accumulate any stress metabolite (Miguel and Barroso, unpublished results).

Although the compounds accumulated in elicitor-treated cell cultures are specific for plant cell culture and rarely affected by the elicitor, differential qualitative and quantitative response to different stress agents have been reported [26].

Elicitor concentration also affects the response intensity of the cell cultures. In general, two types of dose-response curves have been reported: a saturation curve (over-dosage does not show



adverse effects) and, more frequently, a curve with a sharp optimum [26]. The study performed with cell cultures of *Hyoscyamus albus*, treated with different concentrations of *Candida albicans* autoclaved cells (0-6 mg of elicitor/ml of cell suspension), showed a dose-response curve with an optimum at 0.4 mg / ml (Miguel and Barroso, unpublished results).

#### ***Time course accumulation of secondary metabolites***

The time course accumulation of secondary metabolites in elicitor-treated cells is characteristic of the cell culture. Likewise, the activation of a biosynthetic pathway is plant specific, although the amount and the type of the elicitor may affect the time course of product accumulation.

Although the maximum accumulation of secondary compounds, following elicitation, is found after 10 h to 5 days of elicitor treatment, in some cases a minimal exposure time of 20 min was enough for induction of a lasting effect [26, 46].

In a cell line, the time course accumulation may differ for different compounds. In this context, the accumulation of lubimin and 10-*epi*-lubimin in cultures of *Hyoscyamus albus* was maximum 48 h after exposure to the elicitor, while rishitin and solavetivone attained their maximum concentration 96 h after elicitation (Miguel and Barroso, unpublished results).

#### ***Importance of growth stage of the cell culture and cell line***

Accumulation of secondary metabolites in elicitor-treated cells is, in most of the systems, affected by the growth stage of the culture. With some exceptions cell cultures generally respond to elicitation during the growth phase, in particular during deceleration of growth.

The growth stage of the culture may affect qualitatively and quantitatively the products accumulated. According to Eilert [26], *Phythium* culture homogenates stimulated the formation of N-acetyltryptamine in 5-day-old *Catharanthus roseus* cultures, while 10-day-old cells, in contrast, accumulated a whole spectrum of monoterpene indole alkaloids.

Several authors have shown that the addition of the elicitor to a cell culture during its growth phase can lead to an instantaneous, temporary or permanent arrest of cell growth. This rapid change of the physiological stage seems to trigger the expression of secondary metabolism, that otherwise should be activated later [26].

Cell culture lines derived from the same plant species, or even from the same plant, have shown variations regarding the expression of their biochemical capabilities [15, 28]. According to Eilert [26], from a number of variant elicitor-treated cell lines of *Catharanthus roseus*, only one line accumulated indole alkaloids [11] suggested that the contradictory results obtained with elicitor-treated cells of the same plant species may be explained by variance at the cell culture level.

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