Plant Cell Cultures Important Methods for Production of Secondary Metabolites

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Abstract
Plant cell cultures signify possible source of important secondary metabolites which can be used as, nutraceuticals, food additives and medicines. The synthesis of photo-chemicals by the cell cultures in contrast to these in plants is self-governing of environmental conditions and quality fluctuations. In numerous cases, the chemical synthesis of metabolites is not possible or economically viable. Furthermore, the natural food additives are better accepted by consumers in contrast to those which are artificially formed. The secondary metabolites of plants can be identified as compounds that do not play a recognised role in maintaining fundamental life processes in plants, but do play an important role in the plant's interaction with its environment. In the present study, the process of obtaining the secondary metabolites from plant cell cultures is characterized as a multi-stage strategy, and each link would be defined according to specifications of cell cultures or products. For establishing of great production and fast-growing cell lines, the parent plants should be selected. The expression of synthetic pathways can be influenced by the supply of precursors, ecological conditions and the application of elicitors, and also can be changed by superior treatments such as immobilization and biotransformation. Widely bacteria and fungi have been used for the production of antibiotics or amino acids. Simplification of the methods for product recovery, based on the concept of continuous release of products into the increasing media, will improve the efficiency of bio processing. Chemical or physical influences, such as high electric field pulses, may cause this by affecting membrane permeability. The ability of plant cells as sources of secondary metabolites can be exploited by combined research in the fields of in vitro culture establishment, metabolite synthesis targeting and development of product recovery technologies.

Keywords: Plant cell culture, secondary metabolites, biotransformation, elicitors, exudation, food additives, immobilization membrane permeabilisation

Introduction

Plant cell culture methods represent a potential source of useful secondary metabolites which can be used as food additives (flavours, fragrances, and colorants), nutraceuticals, and pharmaceuticals (Zhong, 2001). Environmental conditions, political and labour instability in the producing countries, uncontrollable fluctuations in crop quality, failure of authorities to avoid crop adulteration, and losses in storage and handling are the problems associated with obtaining secondary metabolites from plants. The production of useful and valuable secondary metabolites from cell cultures is an attractive application. The technology of cell culture was created as a potential method for both studying and generating secondary metabolites in plants (Zhong, 2001). Over the past four decades, research has centred on the use of plant cell cultures for the commercial development of a wide range of secondary metabolites, particular in Japan, Germany and the USA, in the same way as bacteria and fungi have been used for the production of antibiotics or amino acids (Mulabagal and Tsay, 2004). For example, there has incredible achievement in the manufacture of shikonin from cell cultures of *Lithospermum erythrorhizon*, berberine from *Coptis japonica* (Matsubara *et al.,* 1989). and sanguinarine from *Papaver somniferum* (Dicosmo and Misawa, 1995). The present chapter reviews advances in optimising environmental factors for metabolite production through plant cell culture, new developments in plant cell bioprocesses, and emerging research on phytochemical recovery.

Plant cell culture techniques

Secondary metabolites obtained by cell culture methods

Plants are an important part of our daily diet, and for decades, their nutritional qualities have been researched intensively. Of the approximately 30,000 recognised natural products, more than 80% are of plant origin (Phillipson, 1990). Which is estimated to be almost four times greater than in the microbial kingdom? Humans have been using plants as a source of carbohydrates, proteins and fats for food and shelter for millennia. Higher plants synthesise a wide range of secondary metabolites in addition to important primary metabolites. The secondary metabolites of plants can be identified as compounds that do not play a recognised role in maintaining fundamental life processes in plants, but do play an important role in the plant's interaction with its environment. Mostly, they have an ecological function as pollinating insect attractants or as defensive mechanisms against predators. The distribution of secondary metabolites in plants is far more limited than that of primary metabolites, only a few species or even a few varieties within a species frequently find a compound. The production of these compounds is often poor (less than 1% DW) and depends greatly on the physiological and developmental stage of the plant species (Namdeo, 2007).

Applications of plant cell culture

It is difficult to grow many plants containing high-value compounds (Rates, 2001). At the same time, because of their highly complex structures and specific stereo-chemical features, the chemical synthesis of plant-derived compounds is often not economically feasible. A desirable alternative to the extraction of the whole plant material is the processing of useful secondary metabolites in plant cell cultures. In the late 1930s, plant cell cultures were first created. However, it was only in 1956 that Pfizer Inc. filed the first patent for cell culture processing of metabolites (Ratledge, 1992).Isolated from cell cultures than from the whole plant (Berlin *et al.,* 1996). were greater amounts of visnagin and diosgenin (Zenk, 1978), showed the excellent metabolic capabilities of plant cells in 1978 and illustrated the spontaneous variability of the biosynthetic capability of plant cells. To define high-yielding cultures for use on an industrial scale (Benjamin *et al.*, 1990). This natural variability is used. Research and development in this field has seen a high rise in the number of patent applications filed since the late 1970s. In1983, shikonin was first developed on an industrial scale by Mitsui Petrochemical Industries Ltd. in plant cell cultures (Fujita and Tabata, 1987).

Plant cell culture currently has direct commercial applications as well as utility in fundamental cell biology, genetics, and biochemistry research.

The application of plant cell culture has three main aspects:

1. Model system for researching genetics, physiology, biochemistry, and pathology of plant cells.
2. Production of secondary metabolites- as a source of products, growth in liquid culture.
3. Breeding and genetics:
    - Micropropogation-to create large numbers of similar individuals using meristem and shoot culture.
    - Selection-screening of cells for beneficial characters rather than plants.
    - Crossing distantly related species by protoplast fusion and regeneration of the novel hybrid.
    - Generation of diploid plants from haploid cultures for faster attainment of homozygous lines in breeding programmes.
    - Transformation, accompanied by either short-term genetic construct testing or transgenic plant regeneration.
    - Virus elimination by propagating from meristematic tissues.

Application for production of secondary metabolites

Cultured plant cells often produce different quantities with different secondary metabolite profiles when compared with the intact plant, and these quantitative and qualitative characteristics can change over time (Tepe and Sokmen, 2007). As shown in **Table 1**, a

higher titer can accumulate certain metabolites in plant cell cultures compared to those in the parent plants.

Table 1: Product yield from plant cell cultures compared with the parent plants

Product	Plant species	Yield	Culture plant (% DW)	Culture/ plant	References
Ajmalicine	*Catharanthus roseus*	1.1	0.4	3.2	Lee and Shuler, 2000
Anthraquinones	*Morinda citrifolia*	18	2.2	8	Zenk, 1977
Berberine	*Coptis japonica*	12	2	3.5	Fujita and Tabata, 1987
Caffeic acid	*Vanilla planifolia*	0.03	0.05	4	Knorr *et al.,* 1993
Ginsenoside	*Panax ginseng*	27	4.5	6	Matsubara *et al.,* 1989
Nicotine	*Nicotiana tabacum*	3.3	2.0	1.6	Mantell *et al.,* 1983
Rosmarinic acid	*Coleus blumei*	26.5	3.2	8	Petersen and Simmond, 2003
Shikonin	*Lithospermum erythrorhizon*	21	1.4	14	Kim and Chang, 1990
Ubiquinone-10	*Nicotiana tabacum*	0.037	0.004	12.5	Fujita and Tabata, 1987

Suggesting that the production of plant-specific metabolites by plant cell culture instead of whole plant cultivation possesses definite potential (Zhong, 2001). Kim and Chang (1990) showed that shikonin by *Lithospermum erythrorhizon* was accumulated in higher levels in cultured cells than in the intact plants. Similar results were shown by Petersen and Simmonds (2003) in the production of rosmarinic acid by Coleusbluemei. Higher quantities of berberine have been obtained from growing cells of *Coptis japonica* (Fujita and Tabata, 1987). This plant accumulates significant amounts of berberine in its roots in four to six years; similar concentrations could be obtained in four weeks using tissue culture. Hara *et al.,* have isolated a cell line of *Coptis japonica* that contained 13% DW of berberine. This culture produced about 1500 mgl^{-1} of this antibacterial alkaloid in 14 days. There are a number of examples of cultured cells producing metabolites not observed in the plant. Thus, *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid (Szabo, 1999).

Food additives from the plant cell cultures

The reason for the use of metabolites synthesized by the plant cell cultures as food additives is not only that they are difficult or impossible to synthesize chemically, but consumers also more easily accept a natural product than an artificially produced one (Ratledge and Sasson, 1992). Food additives contribute to making foodstuffs palatable and attractive by enhancing or improving their flavor, colour, and texture. Food technologies try to respond to these criteria especially with regard to the texture, taste, and aroma of the foodstuff. The need to have the same taste and aroma in order to suit the consumer tastes makes it compulsory to use additional natural or artificial aromas. Since the late 1950s, the

safety of many food additives for long-term use and use has been challenged primarily by national and international regulatory authorities. At the same time, consumer groups, aware of the inclusion of additives in foodstuffs, have put pressure on government bodies to substitute chemical or artificial additives with natural plant tissue additives or plant cell culture synthesizers (Dornenburg and Knorr, 1997). The most precious additives to food.

Table 2: List of some food additives obtained from plant cell cultures

Product type	Plant species	References
Colours		
Anthocyanins	*Vitis vinifera* *Aralia cordata* *Perilla frutescens*	Curtin *et al.,* 2003 sakamoto *et al.,* 1994 Zhong 2001
Crocin	*Crocus sativus*	Chen *et al.,* 2003
Carotenoids	*Lycopersicon esculentum*	Rhodes *et al.,* 1991
Betalaines	*Beta vulgaris*	Trejo-Tapia *et al.,* 2007
Naphthoquinones	*Lithospermum erythrorhizon*	Kim and Chang 1990
Anthraquinones	*Cinchona ledgeriana*	Rhodes *et al.,* 1991
Flavours		
Garlic	*Allium sativum*	Rhodes *et al.,* 1991
Onion	*Allium cepa*	Rhodes *et al.,* 1991
Cocoa flavour	*Theobromo cacao*	Rao and Ravishankar, 1999
Coffee flavour	*Coffea Arabica*	Kurata *et al.,* 1998
Vanillin	*Vanilla planifolia*	Dornenburg and Knorr, 1996
Pungent food additive		
Capsaicin	*Capsicum frutescens*	Rhodes *et al.,* 1991
	Capsicum annuum	Johnson and Ravishankar, 1996
Sweeteners		
Glycyrrhizin	*Glycyrrhiza glabra*	Rao and Ravishankar, 1999
Thaumatin	*Thaumatococcus danielli*	Rao and Ravishankar, 1999
Stevioside	*Stevia rebaudiana*	Rao and Ravishankar,1999

Aromas and fragrances

A mixture of various compounds is a natural aroma; more than 500 have been found in roasted coffee beans and 200 in apples. Natural flavours are vulnerable to food preservation techniques, such as sterilisation, pasteurisation, freezing, etc. Some aromas are altered by enzymatic or chemical reactions and, if stored for a long time, typically vanish. This is why they have been finding replacements since the end of the 19th century. Artificial aroma used to produce artificial aromas from coal.

Pigments

The use of extra pigments was strongly criticised in the 1970s by consumer groups, as most of the colours are created by chemical synthesis and are unrelated to any material that exists naturally. The biotechnological techniques used to manufacture natural food dyes consist of growing higher cells of plants (Zhong, 2001).

1. Shikonin compounds, such as shikonin and its acetyl and isobutyl shikonin derivatives have accumulated in the Lithospermum erythrorhizon roots. The mass cultivation of Lithospermum erythrorhizon cells to produce shikonin compounds was successfully developed due to a shortage of this plant (Kim and Chang, 1990).
2. The broad group of water-soluble pigments that are responsible for many of the vivid colours in flowers and fruits are anthocyanins. Due to the presence of four pH-dependent forms, they change colour across the ph range: they are red at low pH and they turn blue at pH above six. In acidic solutions are widely used in vibrant soft drinks, sugar confectionary, jams, and bakery toppings. Pure anthocyanins are valued at \$2000kg^{-1}, but crude materials are priced at \$2000kg^{-1}.
3. Crocin, the primary pigment of the stigmatic *Crocus sativus*, is commonly used as a yellow food colorant. Its high price and restricted supply restrict the commercial production of saffron pigment. As a geophyte, through the formation of daughter corms, saffron grows slowly and spreads only through vegetative development. To grow 1kg of saffron stigma, it takes 200000 flowers and over 400h of hand labour. The method of cultivation of plant tissue provides great potential for crocine production (Dougall, 1980).
4. Madders are red dyes from Rubia tinctorum, a perennial plant native to the Mediterranean coastal regions, and its roots have been used in Western Europe as red dyes. Alizarin, purpurine, and its glycoside, ruberythric acid, are the main components of the pigment. Simple alizarin is an orange crystal that is soluble in other solvents and in boiling water. Alizarin has an acidic to neutral pH yellow colour and appears to be reddish with a rise in ph. It is highly resistant to heat and light, which is favourable to the food industry. Through the selection of high-producing cell lines and elicitor application, yellow-pigment-producing cells of Rubia tinctorum were obtained (Vasconsuelo *et al.,* 2004).

Pharmaceuticals from plant cell cultures

Higher plants are a rich source of bioactive constituents used in pharmaceutical company. Drugs such as morphine, codeine, cocaine, quinine, catharanthus alkaloids, belladonna alkaloids, colchicine, phytostigminine, pilocarpine, reserpine, and steroids such as diosgenin, digoxin, and digitoxin are some of the plant-derived natural products.

Table 3 Some plant-derived pharmaceuticals (Rao and Ravishankar, 2002)

Sl. No	**Plants species**	**Product**	**Uses**	**Cost US $ kg^{-1}**
1.	*Catharanthus roseus*	Ajmalicine	Antihypertensive	37000
2.	*Papaver somniferum*	Codeine	Sedative	17000
3.	*Colchium autumnale*	Colchicine	Antitumour	35000
4.	*Orchrosia elliptica*	Ellipticine	Antitumour	240000
5.	*Rauvolfia serpentine*	Ajmaline	Antimalarial	75000
6.	*Papaver somniferum*	Morphine	Sedative	340000
7.	*Taxus brevifolia*	Taxol	Anticancer	600000
8.	*Lithospermum erythrorhizon*	Shikonin	Antibacterial	4500
9.	*Catharanthus roseus*	Vinblastine	Antileukemic	1000000
10.	*Catharanthus roseus*	Vincristine	Antileukemic	2000000

Production of secondary metabolites using plant cell cultures

A huge market value is served by plant-derived drugs. Worldwide, 121 clinically useful prescription drugs are produced from plants, according to Rao and Ravishankar (2002). In addition, 12% of the drugs considered fundamental and necessary by the WHO are extracted exclusively from flowering plants (Rates, 2001). In the USA, plant medicinal use surveys showed a rise from just around 3% of the population in 1991 to over 37% in 1998 (Rao *et al.,* 1999). Biotransformation of isoeugenol to vanilla flavour metabolites and capsaicin in freely suspended and immobilized cell cultures of Capsicum frutescens: study of the influence of b-cyclodextrin and fungal elicitor (Dorenburg and Knorr, 1996; Rao and Ravishankar, 1999). In 2002, in the USA, prescription drugs containing photo chemicals were priced at over US$30 billion (Raskin *et al.*: Kim and Chang, 1990). In traditional medicine, 75% of the world's population depends on plants. **Table 3** lists several plant-derived pharmaceuticals. An example of a high-value drug produced from plant cell cultures is paclitaxel, an anti-cancer drug originally extracted from the bark of 50-year-old Pacific yew trees, *Taxus brevifolia* (Tabata, 2006).

A number of successfully developed and marketed cultures of plant cells produce a high amount of secondary metabolites. This technology, however, is still being developed and, despite the advantages, a range of problems must be solved before it can be adopted for the development of useful secondary metabolites for plants.

Table 4: High yields of secondary products

Sl. No.	Plant species	Product	Yield (%DW)	References
1	*Morinda citrifolia*	Anthraquinones	18.3	Zenk, 1977
2	*Perilla frutescens*	Anthocyanins	8.8	Zhong, 2001
3	*Catharanthus roseus*	Serpentine	2.3	Moreno *et al.,* 1995
4	*Lithospermum erythrorhizon*	Shikonin	14	Kim and Chang, 1990
5	*Papaver somniferum*	Sanguinarine	2.6	Dicosmo and Misawa, 1995

Strategies to increase secondary metabolite production

The goal of the food industry is to develop techniques to enable the development of secondary products from the culture of plant cells to be less costly than the extraction of the entire plant under natural conditions and less costly than the synthesis of the product. The need for biochemical and molecular research on the secondary metabolism of plants has been frequently stressed in the face of having to increase the amount of secondary metabolites in plant cell cultures (Dixon, 2005). Research in this field could contribute to the effective modulation of secondary metabolism and could significantly increase the synthesis of a wide range of compounds using plant cell culture technology, such as alkaloids, flavonoids, terpenes, steroids, glycosides, etc. using plant cell culture technology. The method for obtaining secondary metabolites from the cultures of plant cells can be described as a multi-stage process (**Figure 1**). Each link can be individually or in combination with other processes or treatments optimised.

- The initial step of this technology involves the selection of the parent plant according to its molecular and biochemical characteristics, with particular reference to the high metabolite content required. Any component obtained from any plant species can theoretically be used to induce callus tissue; however, efficient callus development depends on plant species and their characteristics. Dicotyledons are fairly ideal for induction of callus tissue as compared to monocotyledons. Stems, leaves, roots, flowers, seeds, and any other parts of plants are used, but younger and fresh explants are preferable explant materials.
- Afterwards, the selection of cell line becomes important. It includes the establishment of high-producing and fast-growing in-vitro cultures. It is possible to identify cell lines that can produce amounts of compounds equal or even higher than in the plant from which they derive (Benjamin *et al.,* 1990). Moreover, increase of metabolite levels using mutants is possible, and selection of suitable analogues for this purpose could be an important factor in order to produce a variety of products. Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires

production of new genotypes through protoplast fusion or genetic engineering; however, this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression. Use of mutagens increases the variability which already exists in living cells. Furthermore, new molecules, which have previously not been found in plants, can be produced by cell cultures.

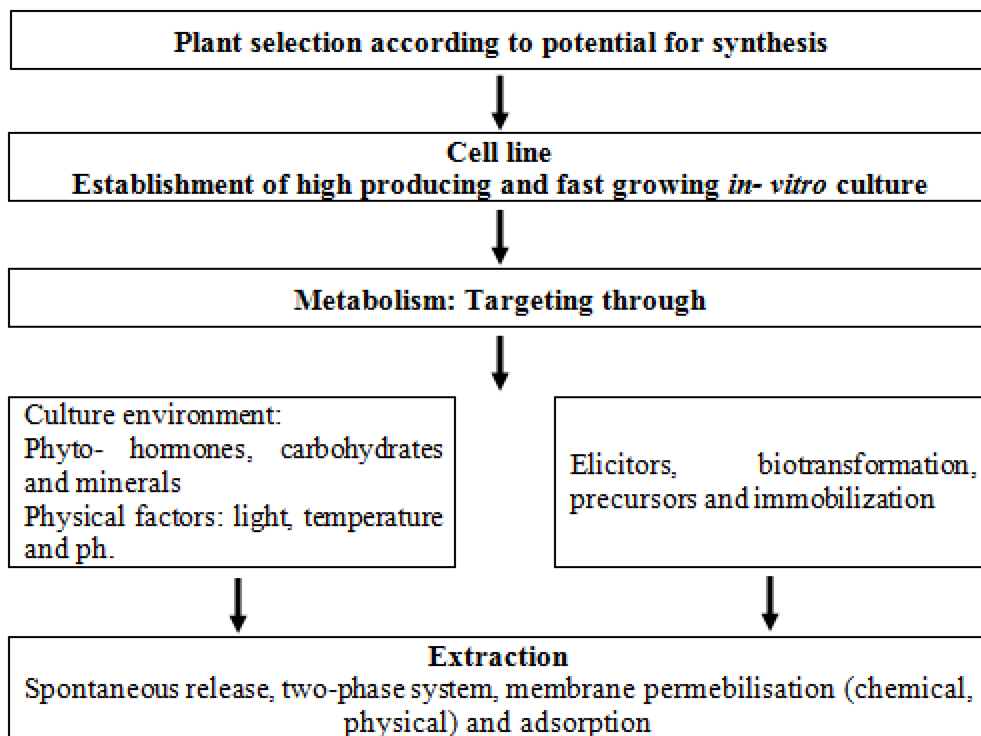

Figure 1: Scheme of secondary metabolite production in plant cell culture

Metabolism targeting

The development of secondary metabolites is influenced strongly by a variety of chemical and physical factors. External influences such as environmental conditions (chemical and physical) and special therapies readily change the expression of many secondary metabolite pathways.

- Plant cell culture medium includes inorganic components, organics, and phytohormones. Changing of medium components (concentration, proportion, and form) is a very powerful way of enhancing the culture efficiency of plant cell cultures. Thus, high auxin level stimulates cell growth, but often negatively influences secondary metabolite production (Gambor, 1995). **Physical conditions**, such as light, temperature, and

medium pH, have also been examined for their effect upon secondary metabolite accumulation in many types of cultures.

- Special treatments include precursor feeding, application of elicitors, biotransformation, and immobilisation. The idea of precursor feeding is based on the idea that supplying compounds that are intermediate or at the beginning of the biosynthetic path gives a good chance of increasing the final product's yield. The production of the desired metabolites is often limited by the lack of particular precursors; biotransformation using an exogenous supply of biosynthetic precursors may improve the accumulation of compounds. Biotransformation is a process through which the functional groups of compounds are modified by cell cultures to chemically different product (Grisebach, 1988). Plant cells can transform natural or artificial compounds introduced into the cultures through a variety of reactions, such as hydrogenation, dehydrogenation, isomerisation, glycosylation, and hydroxylation.
- Plants and plant cells in vitro exhibit physiological and morphological responses to microbial, physical, or chemical factors, which are known as elicitors. Since the secondary metabolites protect plants from the environmental changes, the way to induce their synthesis is to add unfavourable factors, i.e., simulate pathogen attack, herbivores, heavy metals, etc. Elicitation is a mechanism by which plants induce or improve the synthesis of secondary metabolites to ensure their survival, persistence and durability.
- Because of the specialisation of the certain grade of cell, cell immobilisation can result in far higher concentrations of plant cells while hundreds or thousands of them are immobilised in one aggregate. Hydro-colloidal gels, such as alginate and carrageenan, have been used in most of the research in this field to trap plant cells in a gel matrix while allowing easy access to substrates.

Perhaps the most successful bio processing concepts for the development of phytochemicals lead to spontaneous release into a medium where it is easier to retrieve them. One of the most productive research areas for the development of flower-value products that may cause product leakage from cells that normally accumulate the product may be studied. It may also be essential to study the intracellular compartments in which chemical synthesis takes place, as the compounds are transported to the vacuole. Plants often have sites of synthesis and storage of the secondary metabolites in separate cells or organs. Inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium or two-stage system.

Selection according to molecular and biochemical characteristics

The strategies for improving secondary metabolite content in plant cell cultures are the screening and choice of plant species and cultivars rich in useful metabolites. In order to

achieve high-producing cell lines, plants with high levels of the desired products should be used for callus induction.

Plant genotype and cultivar

One of the most significant variables determining the biochemical status of plants and cultures of plant cells is genetic ability. The expression of genes involved in photochemical synthesis can be modified by environmental and physiological influences, but the genetic context is the main determinant. The diversity of genetic potential for the secondary metabolite profile cannot be demonstrated by the Brassicaceae family, which comprises approximately 350 genera and 3500 species and includes vegetables. The comparison of the profiles of main glucosinolates in Brassica vegetables shows that the general content and distribution of sub-classes of glucosinolates is unique for each species. For example, the main glucosinolate in *Brassica juncea* seed is dominated by progoitrin, *Brassica oleracea* seeds contain mainly gluconapoleiferin, whereas *Brassica napus* seeds contain gluconapoleiferin, gluconapin, and glucobrassicanapin (Schreiner, 2005).

Table 6: Distribution profile of glucosinolates in Brassicaceae (Schreiner, 2005)

Plant Species	Glucosinolates (mg 100^{-1}DW)	**Percentage (%) of total glucosinolate**		
		Aliphatic	**Aeromatic**	**Indole**
Brassica rapa L. var. rapa. teltoviensis.	790-890	28	45	25
Brassica rapa L. var. rapa	21-340	43	30	19
Brassica oleracea L. var. italic	40-340	47	8	44
Brassica oleracea L. var. capitata alba	25-275	47	32	20
Brassica oleracea L. var. botrytis	14-280	47	13	39

Obtaining of fast-growing and high-productive cell lines

With inherent genetic and epigenetic heterogeneity, plant cell cultures are often characterised. Cell variability also leads to a progressive decline in productivity and can be related to genetic changes due to culture mutations, or to epigenetic changes due to physiological conditions. The quest for a desired cell population from the heterogeneous ones, usually presented in plant cell cultures (Dornenburg, 1997). May reverse these undesirable changes. Methods of cell cloning provide promising methods of selecting cell lines that produce increased product levels. The physiological characteristics of individual plant cells are not necessarily standardised. For instance, cell aggregates that produce pigments usually consist of producing cells and non-producing cells. To obtain highly efficient cell lines, the variation in the biochemical activity existing within a cell population has been exploited. This is analogous to the isolation of monocolonic bacteria. Matsubara *et al.,* (1989) obtained strain that grew faster and developed a higher amount of berberin, for

example, by cell cloning using cell aggregates of *Coptis japonica*. The selected cell line of *Coptis japonica* produced six times higher amount of berin during the three weeks of cultivation, particularly 1.2gl^{-1}, as primary callus. This selected strain was very stable, producing a high level of berberin even after 27 generations. As shown in **Table 7**, the *Euphorbia milli* strain accumulated about sevenfold the sum of *Euphorbia milli.*

In cultures of *Lithospermum erythrorhizon*, extensive screening of a number of clones resulted in a 13-fold to 20-fold increase in shikonin production (Kim and Chang, 1990). *Lavendula vera* cells grown in the light accumulated a high level of free biotin (Misawa, 1985). To select a high-producing cell line, pimelic acid, a precursor of biotin, was used. The level of biotin accumulated by a selected cell line was 0.9μgl^{-1}, which was ten times the amount found in the leaves.

Table7 Influence of cell cloning on productivity of plant cell cultures:

Plants	**Products**	**Factors (increase of production)**	**References**
Vitis vinifera	Anthocyanins	2.5-4	Curtin *et al.,* 2003
Coptis japonica	Berberine	2.5-6	Matsubara *et al.,* 1989
Lavendula vera	Biotin	9-10	Misawa, 1985
Nicotiana tabacum	Ubiquinone-10	15-179	Dicosmo and Misawa, 1995

A variety of strains of *Nicotiana tabacum* containing high levels of ubiquinone-10 (Dicosm 1995). Have been isolated by Japan Tobacco Inc. A strain was selected from approximately 4000 tested cell clones after the 13th reclamation. The titer for ubiquinone-10 was only 0.36mg g^{-1} DW when *Nicotiana tabacum* BY-2, a parent strain used for cloning, was isolated; thus, the level was increased by selection up to 5.2mg g^{-1}, which corresponded to 180 times the amount provided by the parent plant. Cell cloning is a very useful technique to increase the level of secondary metabolites. However, it is not obvious why cultures contain both high- and low-yielding cells. Kim and Chang (1990). Indicated that the lack of specific enzymes represents the most important reaction for the inability of plant cell cultures to produce secondary metabolites. Cell cloning is a very useful technique to increase the level of secondary metabolites. However, it is not obvious why cultures contain both high- and low-yielding cells. Kim and Chang (1990) indicated that the lack of specific enzymes represents the most important reaction for the inability of plant cell cultures to produce secondary metabolites.

Protoplast fusion

Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires production of new genotypes through protoplast fusion, but this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression once introduced in the plant cells. This suggests that use of

mutagens to increase the variability already exists in living cells. (Sakamoto *et al.,* 1994) reported about the visual selection of *Euphorbia millii*. This procedure was repeated 28 times and one of the cells was determined to produce 1.32% DW anthocyanins in the cells. The levels of the pigments in flowers and leaves were 0.28% and less than 0.01%, respectively.

Industrial production of useful biochemicals by plant cell cultures

With technological progress in the future, plant cell culture will have a greater contribution to the market. For example, the current world market of raw materials of ginseng is about one billion US$ (Zhong, 2001); while cell-cultured Panax ginseng occupies less than 1 percent of the market, its share will increase greatly with enhancement of the culture productivity. The industrial-scale development of shikonin by *Lithospermurn erythrorhizon* cell cultures is provided by Mitsui Petrochemical Industries Ltd. Two stages were involved in the process, plant cells are first grown in a 200-l bioreactor and the resulting biomass is then transferred to a second bioreactor in which shikonin synthesis is favoured by the composition of the crop medium. The productivity of cell cultures is 60 mgg^{-1} per week, 1000 times greater than that of plant roots.

The Bio-organic Division of the Bhabha Atomic Research Centre (India) is carrying out research on mass cultivation of selected cell lines of Rauwolfia serpentina (ajmaline, reserpine), *Papaver somniferum* (thebaine, codeine, and morphine), *Artemisia annua* (artemisinin), and other plant species (Dicosmo, 1995).

Conclusion

Advances in biotechnology, particularly in the cultivation of plant cell cultures, can provide new means to commercially process even rare plants and the chemicals they supply. The benefit of this technique is that it can finally provide a constant, consistent supply of natural products. Synthesis of bioactive secondary metabolites, working in a stable environment, independent of soil and temperature, are the main benefits of cell culture. Building on advances in plant science, the use of *in vitro* platinum cell cultures for the manufacture of chemicals and pharmaceuticals has made great strides. Increased use of cell culture as an instrument to generate secondary metabolites would increase product levels. Increased levels of natural products for medicinal purposes, combined with low product yields and plant harvest supply issues, have revived interest in large-scale technology for plant cell culture. These findings illustrate the ability of in vitro plant cell cultures for the commercial production of secondary metabolites. The implementation of new molecular biology techniques to generate transgenic cultures and to control the expression and regulation of biosynthetic pathways is also likely to be a major step towards making cell cultures more widely relevant to the commercial development of secondary metabolites.

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