PLANT CELL CULTURES AS SOURCES OF VALUABLE SECONDARY METABOLITES:

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1. INTRODUCTION

Aside from the primary metabolic pathways common to all life forms, some reactions lead to the formation of compounds unique to a few species or even to a single cultivar or variety. These reactions are classified under the term "secondary metabolism and their products known as "secondary metabolites" (Luckner and Nover, 1977). These substances include alkaloids, antibiotics, voltatile oils, resins, tannins, cardiac glycosides, sterols, saponins, etc. Apart from their economic and pharmaceutical importance, many secondary metabolites play ecological and physiological roles in higher plants. It has been suggested that secondary compounds produced by plants are important either to protect these plants against microorganisms and animals, or to enhance the ability of one plant species to compete with other plants in a particular habitat (Bell, 1980).

In spite of substantial advances that have been made in synthetic organic chemistry, plants still remain an important commercial source of chemical and medicinal compounds. Plants are the source of 25 per cent of prescribed medicines (Zenk, 1978) and yield a number of expensive products used by the perfumery and food industries. In most cases, these plants have not been subjected to intensive genetic programmes for the optimum production of their secondary products. In addition, there have been technical and economic problems in the cultivation of these plants. Unfortunately many Third World countries producing medicinal compounds from plant sources are politically unstable, and the supply of crude plant material for processing cannot be guaranteed. Also important problems of uniformity of quality and cost fluctuations exist.

It has been proposed and now widely recognized that many of these secondary metabolites produced by intact plants could be synthesized by cell cultures (Klein, 1960; Puhan and Martin, 1970). The occurrence of secondary metabolites in plant tissue culture is not any longer a subject of mere curiosity, but a focal point of bio-technological activity. Today, those who have been involved in plant tissue culture research have the great satisfaction of seeing the results of their work increasingly applied in industry and agriculture. Some of the expectations that they had ten years ago however have proved to be impractical while new ideas and techniques are being utilized profitably and appear to hold great promise for the future. It was in mid-1970's that "secondary metabolite" became the catchword in plant tissue culture. Plant cell cultures were going to produce everything from plant pigments to rare drugs.....profitably. Large investments were made but the inability of tissue culture to fulfill all these promises set the entire field of research back years. However, the recent progress in two important areas namely the

selection of cell lines or cell types with increased capacity for secondary products synthesis and the development of novel systems in which to exploit the properties of these cells, holds promise and suggests a bright future for development of products from plant tissue culture on a commercial scale.

II. ORIGIN OF PLANT TISSUE CULTURE

Plant tissue cultures are established usually by incubating explants or sterilized pieces of tissue (leaf, stem, root, etc.) on a solid medium containing a full complement of macro- and micronutrients. These elements include N, P., Ca, Fe, Mg, Mn, Cn, Zn, B, Mo, S, K and are usually added to culture media in the form of mineral salts. The medium is supplemented with vitamins, growth substances, and amino acids. The cultures have heterotrophic nutrition with either sucrose or glucose as their primary carbon source. The presence of growth substances such as auxins and cytokinins is generally essential to promote growth but the nature and concentration of the growth regulators varies from plant to plant. Typically, an auxin such as 2,4-dichlorophenoxyacetic acid, 2,4,-D or indole acetic acid, IAA and a *cylokinin* such as kinetin or zeatin are required either singly or in combination.

The growth of cells from the excised tissue is irregular and after a suitable incubation at 20-30°C an unorganised mass of largely undifferentiated cells, a so called callus, develops. Callus cultures may be maintained on solid medium for extended periods with subculturing at 4-6 weeks intervals. Cell suspensions are usually established by excising the callus followed by shaking in a suitable medium. By adjustment of the growth hormones in the medium, it is possible to induce differentiation in tissues and thereby regenerate whole plants.

The growth cycle of callus and suspension cultures typically involves an initial large phase followed by exponential, linear and a final stationary phase. It normally takes 20-25 days for cells to reach the stationary phase. These growth rates are very slow compared witha fast growing bacteria such as Escherichia *coli* but it should be recognized that plant cells are much bigger in size and the volume of a culture plant cell can exceed that of an E. coli by several orders of magnitude. This slow growth rate is a major disadvantage in working with plant cell culture and can be overcome only by developing cells which are capable of effecting biotransformation reactions and conversion of simple precursors into products.

III. HETEROGENEITY OF CELLS IN CULTURE

Plants cells in culture are heterogenous. This heterogeneity is in cell shape, cell size, degree of vacuolation and cytoplasmic content. It is also known that cell cultures give rise to variant subcultures showing different concentration levels of particular secondary metabolite. The variation in shape and size can be seen with callus cultures of *Cassia senna* (Rai, 1978) viewed under a light microscope. The heterogeneity in product formation is best seen in cells of beetroot in liquid suspension. In this it is possible to see cells containing the red beetroot pigment, betacyanin, varying from pale pink to deep red, cells which are yellow due to the presence of betaxanthins and cells which are colourless (Rhodes and Kirsop, 1982).

Underlying this obvious heterogeneity are fundamental genetic variations between cell lines. Polyploid, aneuploid and haploid cells, have all been found in plant tissue culture. The reasons for this heterogeneity are not yet clearly understood. Some workers suggest that the fact that callus tissues are disorganised destroys normal polarity within the tissue and leads to aberrent mitoses. Others have attributed the occurrence of aberrant mitoses to use of compounds such as 2,4-D which have mutagenic properties. This inherent heterogeneity however provides a possible source of variant cells some of which may be capable of high product accumulation. At present, much attention is being given to the heterogeneity of cell cultures and exploiting it to isolate stable high productive cell strains.

IV. BIOSYNTHETIC POSSIBILITIES

When a callus or cell suspension is established from a plant there may be one of the four possibilities:

(1) Production of the same compounds in culture that are produced by the parent plant in natural habitat, at the same or different concentrations

(2) No production at all of the metabolites of the parent plant under the given cultural conditions

(3) **Production of compounds in culture which are not present in the parent plant**

(4) Production of compounds in culture some of which are present in the parent plant while some are not.

Based on these biosynthetic possibilities, a variety of compounds have already been investigated and shown to be produced in plant tissue culture. These

range from alkaloids to allergens, antileukemic and antitumour agents, antimicrobial agents, benzo-compounds, cardiac glycosides and other cardiac active substances, steroids, sterols, saponins and sapogenins, etc. Table 1 illustrates some interesting plant products isolated from cell cultures.

Table 1

INTERESTING PLANT NATURAL PRODUCTS ISOLATED FROM CELL CULTURES

COMPOUND	CLASS OF COMPOUND	PLANT
Ginsenosides	Saponin	*Panax ginseng*
Glycyrrhizin	Saponin	*Glycyrrihiza glabra*
Solasonine	Steroidal Alkaloid	*Solanum laciniatum*
Visnagin	Furochrome	*Ammi visnaga*
Quinones	Anthraquinones	*Cassia tora*
Shikonin	Naphthoquinone	*Lithospermum erythrorhizon*
Protopine	Protoberberine Alkaloid	*Macleaya microcarpa*
Scoulerine	Tetrahydroproto-berberine Alkaloid	*Macleaya microcarpa*
Sanguinarine	Berberine Alkaloid	*Macleaya microcarpa*
Hysocyamine	Tropane Alkaloid	*Datura tatula*
Caffeine	Purine	*Coffea arabica*
Thebaine	Morphinane	Papaver *bracteatum*
Codeine	Morphinane	*Papaver bracteatum*
Ephedrine	Phenylethylamine	*Ephedra gerardiana*
Berberine	Berberine Alkaloid	*Coptis japonica*
Cephalotaxine	Alkaloid	*Cephalotaxus harringtonia*
Harringtonine	Alkaloid	*Cephalotaxus harringtonia*
Sennoside	Glycoside	*Rheum palmatum*

V. OPTIMIZATION OF PRODUCT FORMATION

The concept of product synthesis in plant tissue culture is based on the theory of totipotency. A toti-potent cell is one that is capable of developing by regeneration into a whole organism if provided with proper external conditions. Although in principle one assumes that cells are able to express chemical totipotency as well as the morphological one, in many cases valuable secondary metabolites are either still not, or only at low amounts, produced by cell cultures in comparison with the plants from which they have derived. This is particularly true for meta-bolites that are synthesized only in specific structures of the plant. In such cases, it is frequently necessary for some degree of differentiation to occur before secondary metabolites will be produced in tissue cultures.

EARLIER APPROACHES

Efficient production of secondary metabolites by plant tissues culture is largely dependent on environmental and biological factors. There are several approaches that may be used to produce a culture which will yield appreciable amount of a designated secondary product. Some of the earlier approaches involved optimization of light intensity, temperature and growth hormones for the enhanced tissue growth as well as metabolite production. The effect of light and temperature on growth and metabolite production has been extensively studied. It has been found in a number of tissue cultures that light generally stimulates the formation of compounds including carotenoids flavonoids, and polyphenols (Hahlbrock *et al.*, 1976). In sharp contrast to the stimulatory effects of light, Nagel and Reinhard (1975) have demonstrated that blue or intense white light inhibits the synthesis of terpenoids, whereas these metabolites are produced in the dark. A recent report on the effect of light intensity and temperature on the growth and alkaloid accumulation in callus tissue of *Datura stramonium* indicated that rate of callus tissue growth as well as alkaloids formation increased with increasing light intensity, and while 25°C proved to be the most favourable temperature for callus tissue growth, 20°C was the best for alkalid formation (Saleh and Kamak, 1982). The type and concentration levels of growth hormones in the medium play a key role on the yields of desired compounds. There are numerous examples of the alteration of yield of secondary metabolites by the conditions of medium composition (Rai, 1984). In a limited number of cases, medium pH, phosphate concentration, and addition of biologicals (e.g. yeast extract or coconut water), have led to alterations in yield of secondary products (Dougall, 1979).

In many stances, an exogenous supply of a biosynthetic precursor in a culture medium may increase the yield of the final product when productivity is limited by the lack of precursor. For example, in *Scopolia* and *Datura* cultures, production of tropane alkaloids can be markedly increased by the addition of tropic acid, a direct precursor (Tabata *et al.*, 1972).

Biosynthetic capability of cell cultures may also be improved by the artificial induction of genetic mutation (Berlin and Widholm, 1978), but available information suggests that there is need for more work on the artificial induction of mutant cells useful for valuable metabolites production.

CURRENT APPROACHES

Some of the current approaches that are being extensively studied for optimization of product formation include selection of plant cell lines, biotransformation processes and immobilization of plant cells. Also much attention is now given to use of plant tissue culture techniques for producing better medicinal plants by clonal propagation from callus cultures.

1. Selection of plant cell lines

This approach involves the exploitation of natural or induced heterogeneity among cells by selecting variant cell lines which produce the desired compounds. This is in contrast to the usual approach where attempts are made to induce the entire "wild type" culture to produce the compound. Table 2 describes the strategy of a selection procedure as developed in various laboratories (Barz and Ellis, 1981).

By chosing high producing plants for the establishment of cell cultures it is expected that the genetic or epigenetic factors which are responsible in the living plant for the high productivity will be preserved and lead to an enhanced level of product accumulation in cell culture. All available data so far indicate that this strategy does indeed result in a higher chance of finding a cell culture strain with a high content of secondary products.

Table 2

STRATEGY FOR SELECTING HIGH YIELDING CELL CULTURES

Analysis of a plant population for individuals with high accumulation of the desired secondary metabolite

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Establishment of callus cultures from high-producing plants

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Conversion of callus into suspension cultures

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Plating of cells followed by analysis of resulting microcolonies for high productivity of secondary metabolite

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Establishment of suspension cultures from high-producing calli; repetition of serial selection until stable high-producing strains are obtained

↙ ↘

Grow the new line in fermentation facility and harvest products

Regenerate plants from the new lines and resume filed production.

An interesting comparison between two important approaches that are used to produce useful metabolites in cultured plant cells has been reported for *Coptis japonica* cells (Endo *et al.* 1982). The two approaches are: metabolic regulation and selection of lines that produce large amounts of metabolites. Improvement and optimization of culture conditions gave a berberine production of up to 5% dry weight using non-selected cell lines, but repeated cloning of selected cell lines produced berberine up to 10.1% dry weight.

During the formation of callus from explanted tissue and subculture of such callus, differences in appearance may become noticeable and may reflect differences in ability to synthesize and accumulate secondary products. Screening of young subcultures for individual high-yielding calli has become an established and successful procedure (Table 3). Table 4 illustrates examples of some metabolites produced in exceptionally large quantities.

Similarly, our work with Cassia podocarpa callus cultures (Rai and Shok, 1982) demonstrate that it is possible to establish high yielding anthraquinone cell lines by a "visual selection procedure". In this method, cells which show the desired colouration are picked out and subcultured until a pure line is established. C. podocarpa callus tissue are primarily composed of brown and grey tissues. The brown and grey colouration is an indication of comparatively high and low levels of pigment formation in the respective tissues, and hence, of the differential capacity of these tissues for metabolite production. The 'visual selection' procedure was based on a repeated selective separation of brown and grey tissues and their successive subculturing. After three such successive transfers at six weeks interval each, a clear difference in anthraquinone pattern emerged in brown and grey tissues. The repeated selection procedure helped in segregating and/or isolating certain cell types which may be called 'homogenous' with respect to least chemical variability. However, the possibility of individual cells within this homogenous mass to re-exert their inherent chemical variability over a long period cannot be ruled out.

Table 3

HIGH-YIELDING CELL LINES OBTAINED BY SCREENING CALLUS SUBCULTURES

SPECIES	PRODUCT	YIELD	AUTHORS
Ammi visnaga	visnagin	0.31	Kaul & Staba, 1967
Dioscorea deltoidea	diosgenin	1.5	Kaul & Staba, 1968
Panax ginseng	ginsenoside	27	Furuya & Nishii 1972
Macleaya microscarpa	protpine	0.4	Koblitz *et al.*, 1975
Morinda citrifolia	anthraquinones	18	Zenk *et al.*, 1975
Papaver somniferum	alkaloids	5.6	Khanna & Khanna, 1976
Nicotiana rustica	Nicotine	0.29	Tabata & Hiraoka, 1976
Daucus carota	Carotene	0.07	Townsley, 1977
Coffea arabica	caffeine	1.6	Frischknecht *et al.*, 1977
Lithospemum erythrorhizon	Shikonin	4.8	Tabata *et al.*, 1978.
Nicotiana tabacum	ubquinone 10	0.2	Matsumoto *et al.* 1981
Hyoscyamus niger	scopolamine	0.002	Yamada and
	hyoscymine	0.02	Hashimoto, 1982
Cassia podocarpa	Anthraquinone	1.8	Rai and Shok, 1982.

Table 4 SUSPENSION CULTURES WHICH PRODUCE METABOLITES IN LARGE AMOUNTS

COMPOUND	SPECIES	METABOLITE % of dwt	REFERENCE
Rosmarinic acid	Coleus blumei	15	Zenk et al., 1977
Jatorrhizine	Berberis stolonifera	7	Heinz & Zenk, 1981
Anthraquinones	Morinda citrifolia	18	Zenk et al., 1975
Shikonin derivatives	Lithospermum erythrorhizon	12	Fujita et al., 1981
Shikimic acid	Galium mollugo	10	Amrhein et al., 1980
Cinnamoyl putrescine	Nicotiana tabacum	10	Berlin et al., 1982

The anthraquinone content in non-selected tissues of C. podocarpa on an optimal growth medium was 1.3% dry weight. The approach to visual selection of high-yielding cell lines resulted in an increasing accumulation of anthraquinones from 1.3 to 1.8% dry weight (Rai and Shok, 1982).

2. Biotransformation

Similar to microbial transformation reactions, plant cell cultures can be used to carry out specific transformations of organic compounds to a more complex and, from pharmaceutical point of view more useful compounds. The main types of reaction observed during biotransformation processes in cell cultures are oxidation, reduction, hydroxylation, methylation, demethylation, isomerization and expoxidation. In this respect cell cultures play a role of "organic chemist".

A single step biotransformation catalysed by *Digitalis lanata* cells has been developed by Alfermann et al., (1980) to hydroxylate B-methyl digitoxin to B-methyl digoxin. Digitoxin and digoxin are both natural constituents of D. lanata plants. Due to its superior pharmacokinetic properties digoxin is the compound most widely used in medicine whereas digitoxin is generally regarded as a highly toxic by-product of the isolation procedure. The basic goal of biotransformation in this stance is therefore the conversion of digitoxin to digoxin by 12 B-hydroxylation. A valuable feature of this biotransformation process is the observation that practically all of the transformed product can be isolated from the nutrient medium and is not stored in the cells.

Another example of biotransformation processes can be given for *Ruta graveolens* cell cultures (Steck *et al.*, 1973). In this case 4-hydroxy-2quinolone a known alkaloids precursor when added to the growth medium .is biotransformed to alkaloids dictamnine and gamma-fagarine. This is an example of both precursor feeding to stimulate product formation as well as of biotransformation.

However, much of the work in trying to induce product formation is empirical in nature and there are still major gaps in knowledge of the enzymes catalyzing most of the reactions in the biosynthetic pathways and of the regulatory mechanisms involved in their control.

3. Immobilization of plant cells

One of the major problems with high producing cell lines has been their relative instability. Many high-yielding cell lines have been found to revert to producing low yields after a short duration. The technique of immobilization of plant cells appear to hold promise in that it is capable of providing certain stability to these cell lines.

Much of the methodology for immobilization, which was originally developed for micro-organisms has been found to be applicable to higher cells in culture. Reinhard and co-workers (1980) have reported on the immobilization of *Digitalis lanata* cells by suspending them in a sodium alginate solution, pelleting and allowing the product to harden. The granules catalysed the conversion of digitoxin to purpurea glycoside A and hydroxylated B-methyldigitoxin to B-methyldigoxin.

Similarly, Brodelius et al. (1979) have studied the effect of immobilization of Catha*ranthus roseus* cells on the duration and intensity of production of secondary products. The cells were immobilised in alginate and the properties in terms of secondary product formation of both freely suspended and immobilized cells were compared. They showed that the synthesis of ajamalicine in *C. roseus* increased by five times by the immobilised cells after 21 days. More than 80% of this production was found in the medium with immobilized cells whereas for the freely suspended cells all the product was retained within the cells. A patent has been issued for modifying products by immobilized plant cells (Brodelius et al. 1980).

Some advantages of immobilized plant cells over cell suspensions are greater cell viability because metabolic inhibitors are removed from the medium, and that a slow growth rate is immaterial.

Also the finding that immobilisation of plant cells not only stabilizes their productivity but also influences the partitioning of their products between cells and medium is both economically important and of fundamental scientific interest. The nature of the effect immobilization has on release of secondary products is not clearly understood. However, it is clear that immobilization provides cells with a micro-environment closer to that of the intact plant than when in suspension or callus cultures.

4. Clonal propagation from callus cultures

Although callus can be maintained in a proliferative state indefinitely, reorganization can be experimentally induced. By adjustment of the growth hormones in the culture medium it is possible to induce differentiation of organs from callus tissue and carry these forward to regenerate entire plants. As the cells of the callus are derived from a single meristem, all regenerated plants should be practically identical. This fact has obvious commercial implications for the production, in a short period of time, of uniform plants derived from a small number of selected plants. In this way plant cultures can be used to improve the cultivation of these plants.

In practice, genetic changes may occur in cells during their artificial culture, and careful manipulation of the medium is necessary. However, it has been shown, that for Datura innoxia culture, that on differentiation it is the normal 2nd plants, that are favoured (Geier and Kohlenbach, 1973).

VI. CONCLUSION

There have been major developments in the improvement of high-producing strains of cultured plant cells and in the introduction of immobilized systems. These give rise to optimism in the field of plant tissue culture that after much disappointment in the past, systems for production of valuable secondary metabolites will become a practical and economic reality within the next few years.

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