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Plant-Cell Culture: Natural Products and Industrial Application

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Introduction

Plants are notable for their great synthetic versatility: the spectrum of chemical structures synthesized by the plant kingdom is broader than that of perhaps any other group of organisms. It is the possibility of harnessing this synthetic capacity to commercial and social benefit that has provided much of the driving force behind present developments in plant-cell-culture technology. In consequence the last twenty years or so have seen major advances in plant-cell technology directed towards the synthesis of natural products. For many years plant-cell biotechnology was viewed very much as an academic or intellectual exercise which, while providing useful information on the biochemistry and physiology of plant cells, had little commercial potential. However, with increasing interest from industry, particularly the pharmaceutical and food-additive sectors, the position has been transformed. Today there are two processes operating in Japan for natural-product synthesis using plant-cell cultures and at least four more processes are either at, or approaching, pilot plant level in other parts of the world.

In spite of such progress there are, however, still many 'gaps' in our knowledge of the physiology and biochemistry of plant cells and in the ways in which we can manipulate their activities to meet industrial needs. Many of these 'gaps' will need to be addressed as a matter of urgency if plant-cell culture is to make a major impact upon the synthesis of natural plant products.

This review has been written with two objectives in mind: first, to provide a résumé of the 'state of the art' of plant-cell culture, and secondly to pinpoint those areas where major progress must be made if, in the not-too-distant future, further commercial application is to be achieved. Before delving into these two areas, however, it is appropriate to survey in general terms the present range of plant natural products.

Biotechnology and Genetic Engineering Reviews – Vol. 2, October 1984
0264-8725/84/02/41-27$10.00 + $0.00

Plants and plant natural products

For centuries man has used the plant kingdom as a major source of chemical products, be they single substances or complex mixtures. Plants synthesize a tremendous array of chemical structures, a significant number of which possess interesting and sometimes commercially useful chemical and biological properties. Many of these compounds come under the heading of 'secondary metabolites', loosely defined as those substances not essential for the survival of the plant, or being involved in metabolic sequences much removed from the primary or central pathways of the cell. Such a definition excludes substances such as simple sugars and related primary amino acids.

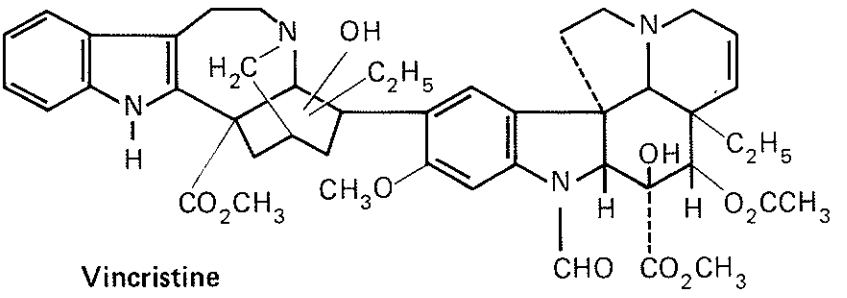
Although typically of low-to-medium molecular weight, many plant products are structurally complex (for examples *see Figure 1*) and in spite of advances in techniques of synthetic organic chemistry, the field-grown plant still provides the best source. Either the compounds are too complex to produce synthetically, or the cost of synthesis is too high to stand comparison with conventional sources. The range of industrial sectors which utilize plant products in one form or another is wide and includes pharmaceuticals, foods and drink, cosmetics and agrochemicals; some examples are given in *Table 1*.

As well as being a key source for certain speciality chemicals, the plant kingdom is potentially an 'Aladdin's Cave' of novel structures for both current and new applications. There are at least 250 000 species of plants on earth, and new ones are continually being discovered and designated, particularly in relatively unexplored parts of the world such as South America. Few of the known species have been examined in any detail for their chemical components: for example, only about 2500 species have been thoroughly screened for pharmacological activity. Consequently the possibilities of finding novel, perhaps commercially interesting, structures are high. As evidence of this, some 1500 new structures are reported from plants each year, of which anything up to one-third may have some degree of biological activity.

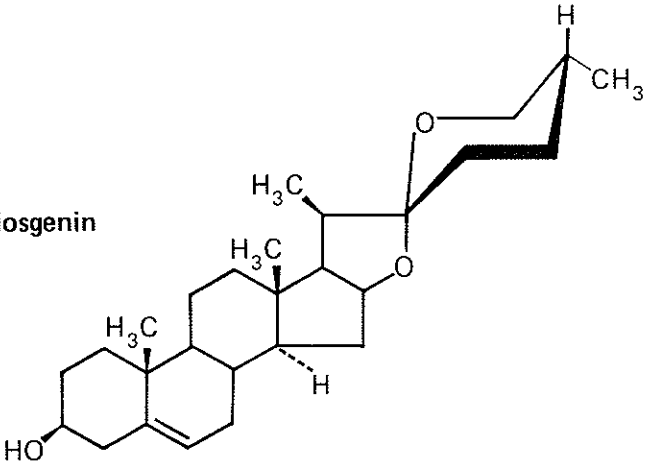
Plant products are typically of low-to-medium molecular weight, ranging from sugars through to complex polymers. In general, from a market viewpoint, they are of low market volume/high cost. In many cases the actual

Table 1. Plant products, applications and origins (after Fowler, 1983a)

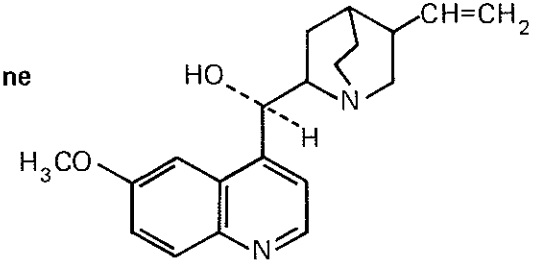
Product	Application	Source
Codeine	Analgesic	*Papaver somniferum*
Diosgenin	Antifertility agent	*Dioscorea deltoidea*
Quinine	Antimalarial	*Cinchona ledgeriana*
Digoxin	Cardiatonic	*Digitalis lanata*
Scopolamine	Antihypertensive	*Datura stramonium*
Vincristine	Antileukaemic	*Catharanthus roseus*
Pyrethrin	Insecticide	*Chrysanthemum cinerariaefolium*
Quinine	Bittering agent	*Cinchona ledgeriana*
Thaumatin	Non-nutritive sweetener	*Thaumatococcus danielli*
Jasmine	Perfume	*Jasminum* sp.



Vincristine



Diosgenin



Quinine

Figure 1. Examples of commonly used plant products. Vincristine: a dimeric alkaloid used in cancer therapy and extracted from *Catharanthus roseus*; diosgenin: a steroid precursor used in oral contraceptives and extracted from *Dioscorea deltoidea*; quinine: an alkaloid used both as an antimalarial and a bittering agent in soft drinks, extracted from *Cinchona ledgeriana*. Redrawn from Fowler (1983b), courtesy of *Biochemical Society Transactions*.

end-product may not be a single highly purified substance, such as is found for instance in many pharmaceuticals, but a mixture or blend of a number of active principles. This latter situation applies particularly to perfumes, flavours and aromas, but also includes such complex materials as cocoa butter fat and cooking oils. The chemical complexity, and hence potential variability, of many of these plant products has long been a point of concern to manufacturers in this area. This has manifested itself in the drive to define the various active principles in some of the more complex materials in an attempt to improve, in particular, product quality. In spite of such problems, and mainly because of economics, the plantation crop has still proved to be the most effective means of synthesis. The key areas which utilize plant products are outlined below.

MEDICINALS

An abundant folklore exists regarding the use of plants in medicine. Herbal remedies are still used extensively in many parts of the world, particularly Asia and South America. Additionally, however, a large number of highly purified substances from plants are used in modern medicine covering a wide spectrum of pharmacological activity. It has been estimated that in the USA some 25% of prescribed medicines are derived from plants; a similar picture is thought to be true of Europe. The ten most prescribed current drugs are listed in *Table 2*, together with their pharmacological activity and origin.

Table 2. The ten most prescribed medicinals from plant sources

Medicinal agent	Activity	Plant source
Steroids from diosgenin	Antifertility agents	*Dioscorea deltoidea*
Codeine	Analgesic	*Papaver somniferum*
Atropine	Anticholinergic	*Atropa belladonna*
Reserpine	Antihypertensive	*Rauwolfia serpentina*
Hyoscyamine	Anticholinergic	*Hyoscyamus niger*
Digoxin	Cardiatonic	*Digitalis lanata*
Scopolamine	Anticholinergic	*Datura stramonium*
Digitoxin	Cardiovascular	*Digitalis purpurea*
Pilocarpine	Cholinergic	*Pilocarpus jabonandi*
Quinidine	Antimalarial	*Cinchona ledgeriana*

At the turn of the nineteenth century, plant extracts, concotions and potions provided the general practitioner with his main armoury against disease and sickness. In spite of the development of synthetic drugs and microbial products, the plant kingdom still provides a range of key drugs. Currently, there is also increased interest in the plant kingdom as a source of novel substances with potentially useful pharmacological activity.

Plant products used as pharmaceutical agents display great diversity in both chemical structure and pharmacological activity. Some examples of this diversity are illustrated in *Table 2*. Plant-derived drugs are typically potent in their action: they are highly efficacious at low concentrations, but can rapidly

produce dangerous side-effects and even death at slightly increased or sustained dosage rates. This has, of course, manifested itself through the use of the plant kingdom as a major source of poisons (*see later*).

Closely associated with pharmaceuticals are narcotics and stimulants. The plant kingdom has a long history as a source of these substances. From a medicinal viewpoint the most important are the analgesics, codeine phosphate and morphine sulphate, both alkaloids which are derived from the opium poppy *Papaver somniferum*. Although both of these substances are highly efficacious as pain killers, chemically near relatives, such as heroin, are a cause of major concern to legislative and law-enforcement agencies around the world. These highly addictive and potentially lethal compounds, together with others such as marijuana, cannabis and cocaine, are the cause of much misery and suffering.

At a less insidious but none the less important level come tobacco and nicotine. The use of tobacco, although declining in some parts of the world, is marked by the dependence of many smokers on the high nicotine content of cigarettes. Stimulants are not generally harmful but do, in some cases, lead to a mild form of addiction. Most of the stimulants are to be found in beverage form and relate to the use of caffeine or its near relative theobromine. Caffeine is particularly widely distributed world-wide and occurs in a surprisingly large number of plants. The two common ones are *Camellia sinensis* (tea) and *Coffea arabica* (coffee). Other stimulants are known to occur, particularly in parts of the Far East and South America. These tend to be used by local tribes and their precise basis of action and effect is little understood.

On a more sinister note the plant kingdom has for centuries been used as a major source of poisons. There are many historical examples of their use: for instance, the great philosopher Socrates died of hemlock poisoning, and the Italian Borgia family of Venice became all-powerful through the sophisticated use of a whole range of plant-derived poisons. More positively, plant-derived poisons are still used today by often primitive people in undeveloped parts of the world to catch animals for food. The use of curare by the tribes of South America is a good example. Many plant-derived poisons are potent neurotoxins, such as ricin from castor bean. Additionally, they tend to be extremely active at low concentrations, their speed of action ranking with poisons from the most venomous species in the animal kingdom. If they can be suitably controlled they may be of potential use as new biocides.

FINE CHEMICALS

This is a heading which covers a tremendous range of both chemical structure and biological activity. The plant kingdom provides a whole range of products classified as fine chemicals and covering such areas as perfumes, aromas, flavours, colourants and food additives. Fine chemicals range from single highly purified compounds to complex mixtures and blends. They include products of very high cost and low market volume as well as bulk products of low value. For instance in the perfumes, costs may be in the region of US$5000/kg upwards, with market volumes of only perhaps tens of kilograms.

In contrast, substances such as cocoa butter fat, a basic constituent of chocolate, costs only about US$4000/t with a market volume of some 20 000 t. The range of chemical structures found in each of the speciality chemicals areas, again testifies to the great versatility of the plant kingdom as a resource of chemical structures and biological activities. This whole area is, of course, a crucial one in the potential for new products, particularly in those sectors concerned with aromas, flavours and cosmetics, all of which are very susceptible to the whim of the consumer. Again, it is worth noting the increased interest of the chemical industry in the plant kingdom as a source of speciality chemicals. Given that it now costs US$50–$100 $\times$ 10^6 to develop a new synthetic product with all the problems of toxicological screening etc., it is perhaps not surprising that we are beginning to see a renewed interest in plants as sources of new industrial compounds.

AGROCHEMICALS

In recent years the plant kingdom has become an important source of a major insecticidal product as well as providing synthetic chemists with core structures on which to build new insecticidal agents. The discovery of the pyrethroids in the flowers of *Chrysanthemum cineriaefolium* and the demonstration of their potent insecticidal activity opened up a key area at a time when there was widespread concern over the use of DDT and the organophosphates as insecticides. Today, the natural pyrethroids obtained from the chrysanthemum flower buds are used predominantly in domestic insecticide sprays, while the synthetic derivatives are used in general crop spraying. Although both forms are extremely effective as insecticides, the natural pyrethroids do suffer from problems of instability on exposure to light. With the synthetic pyrethroids major questions about their use concern possible problems of insect resistance and carcinogenicity or teratogenicity. Little information is available on either of these aspects at present. The pyrethroids do appear to be one of the most effective and least problematical group of insecticides so far discovered and seem to be assured of a long product life. The other key area of agrochemicals concerns plant growth regulators. There is currently great interest in this area, particularly where the possibility of enhancing crop yields and quality is concerned. To date, however, there has been little exploitation of the plant kingdom as a primary source of plant growth regulators and much of the material so far has come from fungal sources, e.g. gibberellic acid.

Against this background of traditional plantation-derived products it is appropriate to ask what contribution plant-cell-culture technology can make to an already extant industry. Most textbooks and reviews see plant-cell culture as an *alternative* production strategy, and in support tend to quote the following factors:

1. Independence from environmental factors;
2. Defined production system, not limited by seasonal considerations;
3. More consistent product quality and yield.

There may also be advantages in downstream processing and recovery of product as compared with conventional technology. However the key point as to whether plant-cell-culture technology is successful on a commercial scale centres on cost comparison with conventional technology and, perhaps more important, whether industry can see an economic advantage. There are slight exceptions to this, in that plant-cell cultures may be a route to novel products, in which case other considerations become paramount. This will be discussed again later.

Plant-cell culture: historical perspective

While a great deal of progress has been made in the science and technology of plant-cell culture during the last twenty years or so, the origins of plant-cell and tissue-culture techniques lie back in the nineteenth century. Haberlandt in the late 1890s (Haberlandt, 1902) was probably the first person to take a major initiative in the area. He was able to isolate cells from a number of higher plants and to maintain them in a viable state in simple nutrient solutions. However, he did not at this stage observe major cellular growth or cell division. After the turn of the century a number of workers became interested in plant-cell culture, and reports of the establishment of growing and dividing cell cultures began to appear. Progress was, however, slow, principally because of the exceedingly low growth rates and long division times associated with plant cells.

In the 1930s a particular breakthrough occurred in that it was found possible to regenerate whole plants from cell and tissue cultures. This work paved the way for present developments in the large-scale micropropagation of plants using tissue-culture techniques (for summary *see* Murashige, 1978). Although a few references to the possibility of using cell cultures as a system for natural-product synthesis began to appear at around this time, it was not until the 1950s that Routier and Nickell (1956) laid out the case in detail in a Pfizer (US) patent application. Following this, the pace began to quicken as more and more species were brought into culture and screened for desired natural products. Success in initiating cultures was not in general, however, accompanied by success in establishing cell lines that synthesized products. A survey of the literature up to about 1972/73 reveals very few cell lines capable of synthesizing commercially interesting products characteristic of the parent plant. However, even where synthesis occurred, specific product concentrations were exceedingly low, often barely existent. In the latter part of the 1970s the situation changed substantially: an increasing number of reports began to appear of cell-suspension cultures which synthesized desirable products, occasionally at levels approaching, or even higher than, those in the intact plant.

The 1970s were also marked by developments in large-scale growth and process technology for cell cultures. Japanese workers explored the growth of tobacco cells in 20 m^3 conventional stirred tank reactors (CSTRs) and, in Germany, air-driven reactors were investigated for their applicability as a large-scale growth system for plant cells. A number of laboratories also began to explore immobilized plant-cell systems, while other groups turned to more

fundamental questions of cellular physiology and biochemistry and relationships between growth and natural-product biosynthesis. A quickening of the pace of research into plant-cell biotechnology is apparent during the last decade. This can be well seen in the reviews and reports of the 1974, 1978 and 1982 International Congresses on plant-cell culture (Street, 1974; Thorpe, 1978; Zenk, 1978; Fujiwara, 1982). An attempt has been made in *Table 3* to present something of the time perspective in the development of plant-cell cultures.

Table 3. The development of plant-cell culture

Date	Progress
1890s	Haberlandt first established plant cells in culture.
1930s	Regeneration of plants from cultures.
1950s	First large-scale culture of plant cells.
1960s	Development of protoplast fusion techniques.
1970s	Isolation of cell lines synthesizing significant levels of desired products. Japanese large-scale tobacco process.
1980	Immobilized-cell technology. Further development of natural-product-synthesizing systems.
1983	Processes for shikonin and berberine production on stream in Japan.

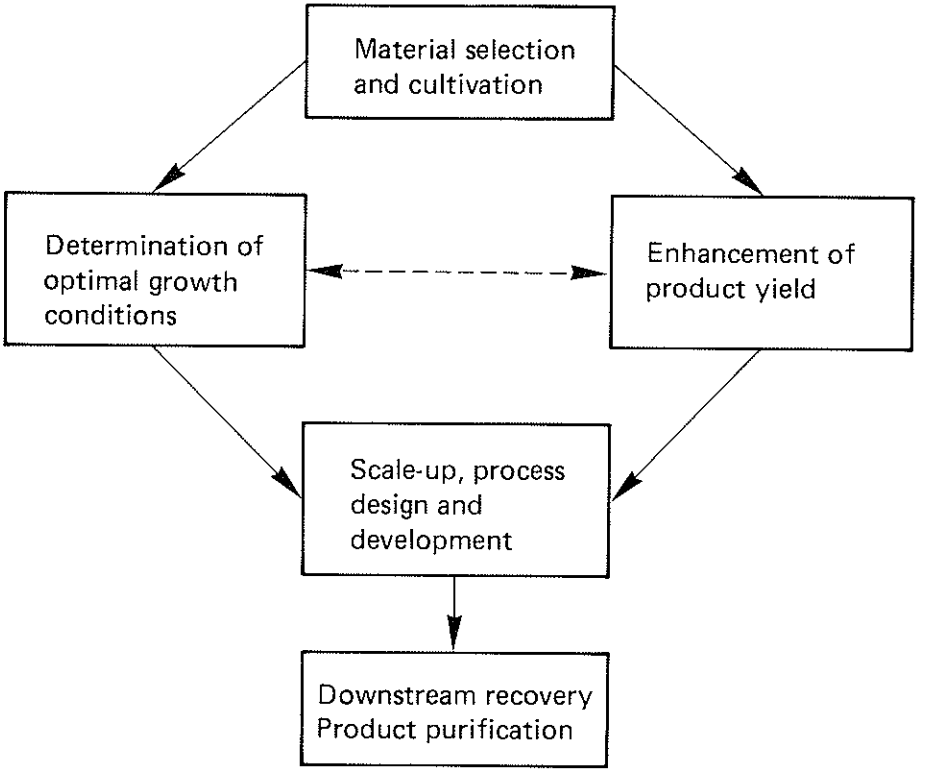


Figure 2. Stages in the development and operation of a plant-cell biotechnology process.

With this widespread background of information, it is now possible to lay out the general strategy usually to be adopted in developing a plant-cell biotechnology process (*Figure 2*). This strategy will form the framework for the rest of this review.

Towards a plant-cell biotechnology process

CULTURE INITIATION AND MEDIA FORMULATION

The statement that it is now possible to establish any known plant species in culture must, however, be qualified immediately by saying that the time required and manipulations necessary vary tremendously from species to species. Some species, e.g. carrot, enter into culture very quickly without any major problems; other species, such as many of the cereals, have been found to take much longer and often require highly specialized growth conditions. A key point in culture initiation is to begin with healthy, disease-free tissue and then to have an effective technique for removing microflora from the tissue before culture initiation is attempted. Methods of culture initiation are dealt with in detail by Street (1977).

An important development in the methodology of plant-cell culture in recent years has been the formulation of defined media. Plant-cell media are complex and contain:

1. Macronutrients (e.g. K^+, Na^+, Cl^-, PO_4^-);
2. Micronutrients (e.g. Al^{2+}, B^{2+}, CO^{2+});
3. Carbon source (e.g. sucrose, glucose);
4. Nitrogen source (e.g. NO_3^-, NH_4^+, glutamate);
5. Growth factors (e.g. kinetin, auxin).

A variety of media formulations exist, some of which have become 'standard' (Gamborg *et al.*, 1976). It is in the area of growth-factor addition that major progress has been made. For many years (and still in a number of cases) coconut milk (the liquid endosperm of the coconut) was a key growth requirement. Coconut milk contains a number of growth promoters together with other nutrients. Unfortunately it exhibits great variation in both qualitative and quantitative composition, making precise growth, and hence process control, difficult to achieve. The definition of growth-promoter requirements for many cell lines has overcome this problem in specific instances.

Media formulation affects not only culture initiation but also growth rate, biomass formation (in productivity terms) and specific product synthesis. As stated earlier, the majority of products derived from plants are secondary metabolites and their synthesis is typically growth-dissociated: that is, they are produced after cell division and growth within the culture has begun to slow down or even stop, or as the culture moves from log growth into stationary phase. The consequence of this is that culture regimens (including nutrient formulations) which enhance cell growth, division rate and biomass production do not necessarily enhance, and may even reduce, the level of natural-product synthesis.

Before considering the interaction (or lack of one) between culture growth and natural-product synthesis, data relevant to the individual areas should be considered.

GROWTH AND BIOMASS PRODUCTION

Biomass yields in the range 5–20 g dry weight per litre have been recorded by a number of workers. In particular, Kato *et al.* (1977) have recorded yields of 20 g/ℓ and more, from tobacco-cell cultures grown in vessels of 0·015–1·5 m^3 capacity. In our own laboratory, tobacco cells grown in 250 ml shake flasks have achieved biomass levels exceeding 50 g/ℓ dry weight. However, it must be said that at these levels of dry-weight biomass, mixing and nutrient supply to the culture is extremely inefficient (Tanaka, 1981; Fowler, 1983a). The range of species for which good (5–30 g/ℓ) biomass yields have been achieved is also increasing and includes, in addition to tobacco, species as varied as *Catharanthus roseus* (Fowler, 1982; Pareilleux and Vinas, 1983; Smart and Fowler, 1984a,b), and *Dioscorea deltoidea* (Tal and Goldberg, 1982). In both cases a linear relationship between the level of carbon supplied and the amount of biomass produced was observed up to about 6 g/ℓ initial sucrose supply. Increase in the initial sucrose level above about 9 g/ℓ with *C. roseus* cultures (Fowler, 1982) did not result in further biomass accumulation. In this particular case maximum yield was 32·5 g/ℓ.

Carbon conversion rates with sucrose as carbon source are typically in excess of 40% (e.g. *Catharanthus roseus* (Fowler, 1982), 60%; *C. roseus* (Pareilleux and Vinas, 1983), 78%; *Discorea deltoidea* (Tal and Goldberg, 1982), 40%). However, caution must be exercised with such data: incoming sucrose is not only oxidized but also converted into storage polymer as starch (distinct starch grains can often be observed in cell cultures after sucrose feeding). This starch may not necessarily be utilized, even though the culture may subsequently become carbon limited. In consequence, dry-weight measurements may include residual stored carbohydrate polymer which will contribute to the biomass yield figures but which has not been metabolized beyond storage polymer. Such an explanation may account for the very high carbon conversion figure quoted by Pareilleux and Vinas (1983) in their work with *C. roseus* cultures. In contrast are data for other cell systems (e.g. sugar-cane; *see* Fowler, 1982), where starch accumulation as described above does not occur, but which show conversion rates of around 55% at final biomass yield.

Attempts have been made to grow plant cells in suspension culture on a wide variety of carbon sources, including refined sources such as glucose, fructose, galactose, sucrose, mannose and lactose, and relatively unrefined sources such as starch, milk whey, molasses and sugar syrups (Fowler, 1982; Sarkissian and Fowler, 1984). Although growth on some of the less common refined sources (e.g. mannose and lactose) does occur, it is much restricted compared with sucrose or glucose. A similar situation exists with the non-refined sources: in the case of lactose and starch this appears to be due to low activity of β-galactosidase and the amylase/debranching enzymes respectively in the nutrient broth. This is unfortunate, in that the ability to use a low-cost 'crude'

carbohydrate substrate would make a major contribution to the area. A simple cost calculation shows that the carbon source is probably the most expensive individual item in the nutrient medium.

In addition to the carbon source, other factors have been shown to have an important and probably direct impact on biomass production: these include the level of aeration and the nitrogen source. Observations by Kato, Shimizu and Nagai (1975) with tobacco cells indicate a close relationship between the initial gas transfer coefficient (K_La) and biomass yield. At low K_La values (5/ℓ or less) Kato and co-workers observed a marked limitation on biomass yield. For K_La values of between 5 and 10/h, the relationship between increasing gas transfer coefficient and increasing biomass yield was linear. Similar data have been reported from studies in our own laboratory with *Catharanthus roseus* cell suspensions (Smart and Fowler, 1984a). In this work, and in contrast to the work with tobacco, a linear relationship between biomass and K_La value was noted up to K_La values of 2/h. Beyond this, a decline in biomass yield was observed, together with a fall-off in productivity. While the work of Pareilleux and Vinas (1983), also with *C. roseus*, shows that oxygen is key in this relationship, their data together with that of others indicates that the level of carbon dioxide is also of crucial importance. This is surprising as none of the cultures involved in any of the above studies is photoautotrophic in nature. However, Nesius and Fletcher (1973), Gathercole, Mansfield and Street (1976) and P. Hegarty, N.J. Smart and M.W. Fowler (unpublished results) have all shown, with different cell lines, that cultured plant cells are able to fix carbon dioxide non-photosynthetically. Furthermore, at high aeration rates carbon dioxide may actually be 'stripped off' from the culture, with a concomitant loss in biomass yield (Smart and Fowler, 1981). Addition of carbon dioxide back to the culture through a bleed system gives significantly enhanced biomass yields (*see* Fowler, 1983a). This is an area much in need of further detailed investigation.

The source of nitrogen, both qualitative and quantitative, has been shown to affect biomass yield markedly (for review *see* Dougall, 1980). However, there are no systematic surveys of the effects of nitrogen source on biomass yield as seen for different carbon sources. Noguchi *et al.* (1977) have shown that the nature and level of the nitrogen source also markedly affects the growth rate, which leads to a consideration of biomass productivity, i.e. g dry wt/ℓ/unit time. Data on biomass productivity are relatively sparse and no systematic study has yet been carried out. However, the data that are available are encouraging from the viewpoint of system scale-up (*see later*). Noguchi *et al.* (1977) working with tobacco cells have reported a productivity of 6·9 g/ℓ/day with a 0·035 m^3 bioreactor, while Kato *et al.* (1976) recorded a productivity of 3·82 g/ℓ/day with tobacco cells grown in a vessel of capacity 1·5 m^3. We have obtained similar results with *Catharanthus* cell suspensions: in this case a productivity of about 5 g/ℓ was achieved in a 0·005 m^3 bioreactor (Smart and Fowler, 1984b).

A number of other factors have been implicated in a variety of effects on biomass yield and productivity (for reviews *see* Martin, 1980; Seibert and Kadkade, 1980). However none have been thoroughly investigated and certainly not to the point where definitive statements could be made about the manipulation of such factors in developing process systems.

PRODUCT YIELD, ITS DEVELOPMENT AND ENHANCEMENT

Of all the facets of process development, this is the most important. Not only is there no process without a product, equally the *level* of product is crucial and must present an advantage both in concentration and productivity terms, compared with a traditional process.

For many years, product synthesis by plant-cell cultures has given cause for concern. As outlined earlier, until the 1970s there were few reports of cultures synthesizing desired natural products and, even where this occurred, levels were exceedingly low. Furthermore, it had become almost a dogma that secondary product synthesis was unlikely unless there was advanced tissue or even organ formation in the culture. The regeneration, from cultures, of plants which produced both qualitatively and quantitatively the same spectrum of products as the plant from which the culture was initiated did, however, serve to demonstrate that the ability for product synthesis was not lost during culture but, for some unknown reason, was not expressed (Dougall, 1979a).

In spite of the apparent recalcitrance of the system there were, nevertheless, indications of the potential. For example Kaul, Stohs and Staba (1969) and Khanna and Mohan (1973) reported quantifiable levels of diosgenin in cell cultures, as did Furuya and Ishii (1972) for ginseng saponins and Reinhard, Corduan and Volks (1968) for visnagin. For reasons largely unknown, the situation changed markedly in the mid-1970s with an increasing number of reports, not only of cell cultures synthesizing substances of commercial potential, but also at levels approaching (and sometimes exceeding) those observed in the intact plant. Some indication of the synthetic potential of plant-cell cultures as known at present may be obtained from the list of

Table 4. Substances reported from plant-cell cultures (after Fowler, 1981)

Alkaloids	Latex
Allergens	Lipids
Anthroquinones	
Antileukaemic agents	Naphthoquinones
Antitumour agents	Nucleic acids
Antiviral agents	Nucleotides
Aromas	
	Oils
Benzoquinones	Opiates
	Organic acids
Carbohydrates (including polysaccharides)	
Cardiac glycosides	Peptides
Chalcones	Perfumes
	Phenols
Dianthrones	Pigments
	Plant growth regulators
Enzymes	Proteins
Enzyme inhibitors	
Flavanoids, flavones	Steroids and derivatives
Flavours (including sweeteners)	Sugars
Furanocoumarins	
	Tannins
Hormones	Terpenes and terpenoids
Insecticides	Vitamins

Table 5. Examples of enhancement in natural-product yield in selected cell lines, compared with parent-plant material (after Fowler, 1983a)

Chemical product	Plant	Cell culture yield (% dry wt)	Whole, (field-grown) plant yield (% dry wt)	Ratio cell culture/whole plant
Glutathione	*Nicotiana tabacum*	1·0	0·1	10
Nicotine	*Nicotiana tabacum*	5·0	2·1	2
Anthroquinones	*Morinda citrifolia*	18·0	2·2	8
Rosmarinic acid	*Coleus blumei*	15·0	3·0	5
Ajmalicine	*Catharanthus roseus*	2·2	0·3	7
Serpentine	*Catharanthus roseus*	1·8	0·5	3
Diosgenin	*Dioscorea deltoidea*	3·5	2·0	2

substances in *Table 4*, while the data in *Table 5* indicate the increased yields so far achieved with products in cell-suspension cultures. However, as pointed out by Dougall (1979b), such data should be treated with some degree of caution. While that obtained from the whole (field-grown) plant is typically from the plant parts containing the highest concentration, data from the cell cultures is an average across the total cell population, and is not therefore strictly comparable. There is of course a further point here. If, as is generally accepted, not all of the cells in a culture are productive, then the actual levels of product accumulating in individual cells could be much higher. For instance if in *Table 5* only 50% of the cells of *Nicotiana tabacum* cultures were synthesizing nicotine, certain individuals could well contain 10%, perhaps more, of the dry weight as nicotine. The problem then becomes one of individual cell selection and cloning (*see later*).

Although an increasing number of cell suspensions have been isolated which synthesize desired products, it is still not possible to define the precise conditions for the development of such systems. Indeed, at least three distinct conditions, all based on morphological associations, can be recognized which *may* result in natural-product synthesis. These include:

1. Where tissue or organ development is, at this moment, an absolute requirement;
2. Where the culture is characterized by some degree of cellular differentiation (although the morphologically distinct cells need not necessarily be the site of product synthesis);
3. Where no signs of morphological development or differentiation are apparent and the culture has an appearance of a group of meristematic cells.

Pictorial examples of the latter two situations are contained in Fowler (1983a).

The strategy increasingly adopted to develop high-yielding cell cultures tends to follow traditional microbial practice, and is outlined in *Figure 3*.

The selection of appropriate material for culture initiation has been a matter of much discussion. Until a few years ago, two totally opposed views were

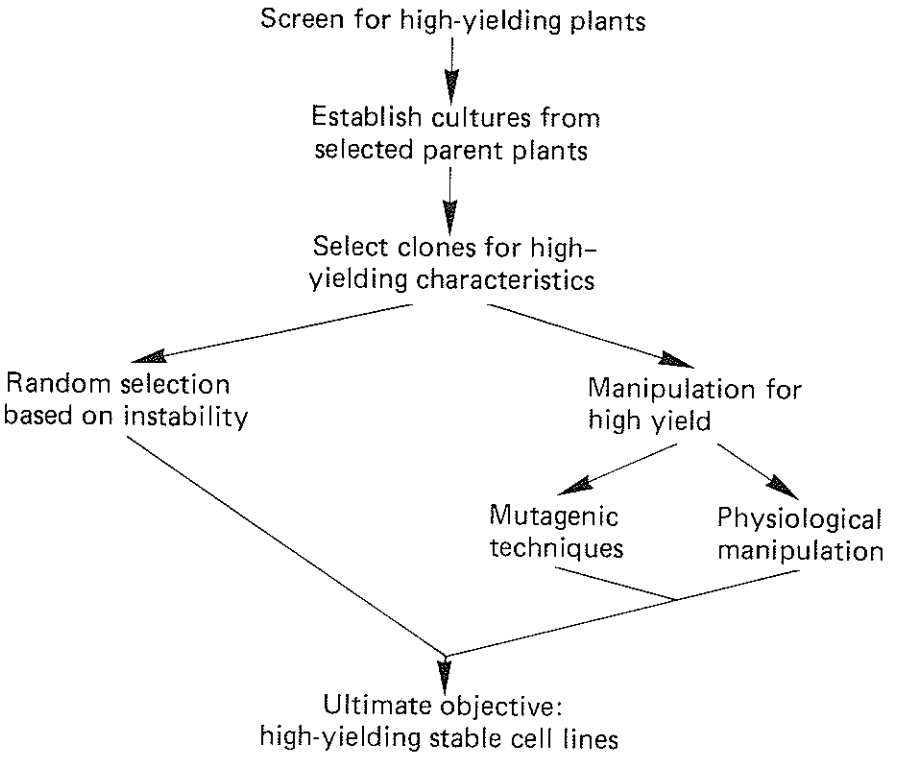


Figure 3. Selection strategy for high-yielding cell lines.

extant: the first maintained that it was necessary to use plants with high product-yield characteristics for culture initiation (*see* for example Zenk *et al.*, 1977); the second maintained that this was unnecessary and that, through selection and cloning, it should be possible to obtain high-yielding cell lines from low-yielding parent plants (*see* for example Doller, 1978). Careful experimentation by a number of workers, Dougall's group in particular (*see* Kinnersley and Dougall, 1980) has shown, however, that the first approach is probably the most effective; i.e. to obtain high-yielding cultures, start with high-yielding parent-plant material.

Once cultures have been initiated, a number of possible approaches then exist to product yield enhancement (*Figure 3*). The classic approach is to screen populations for high-yielding individuals and then to clone and develop further populations. A number of constraints have made this approach difficult for plant-cell culturists. First, the slow growth rate of the cells makes the development of large populations of cells difficult; secondly, cell cloning has only recently become established for plant cells and is still difficult; thirdly, concentrations of desired metabolites in single cells are typically at the limits of detection for classic analytical techniques. Although it is extremely doubtful if the problems of slow growth rate will disappear, techniques for producing

clones are improving and — perhaps more important — the development of radio-immunoassay analysis (RIA) (Zenk *et al.*, 1977) is proving to be of great value for microanalysis. This system has now been applied to a variety of cultures and products with much success. Further developments have come through the use of enzyme immunoassay systems (ELISA). These are under study in a number of laboratories and, in comparison with RIA, have the advantage of speed and the possibility of auto-analysis.

The above approaches rely on the inherent instability of cell lines, which in itself is a cause for some degree of concern. The alternative approach is to manipulate the cell lines physically or chemically, thereby applying a 'selection pressure'. Again, this is an approach which has been successfully applied to microbial systems, and mutagenesis is one aspect of this. This is, however, an area which has been the subject of particular controversy in plant-cell culture circles. Some workers claim that many mutants have been induced using classic chemical and physical techniques, whereas others suggest that no more than two or three cases have actually been substantiated. The use of mutagens and selection of resistant strains is, however, beginning to gain momentum. Studies by Lescure and Peaud-Lenoel (1967) showed at an early stage that *N*-nitrosoguanidine when applied to *Acer pseudoplatanus* cells did result in increased frequency of autotrophic cells. Subsequently Nishi *et al.* (1974) demonstrated enhanced numbers of colonies in *Daucus carota* cell lines with qualitative and quantitative changes in carotenoid content. Since then, other mutagens have been tested and are reviewed more fully by Widholm (1980). However, this whole area is controversial and the effects of mutagens on different cell lines are extremely variable. Nevertheless, some interesting and potentially useful effects have been noted and undoubtedly this is an area worthy of further investigation.

A more detailed literature is available regarding physiological manipulation of cultures aimed at enhancing product yield. As discussed earlier, a key development here has been the formulation of defined media allowing the diagnostic manipulation of media composition aimed at specific effects. A variety of medium constituents have been shown to affect product yield, of which carbon source, nitrogen source, phosphate level and phytohormone content are probably the most well studied. The whole area has recently been reviewed by Mantell and Smith (1983b), and the salient features are discussed below. It should not be forgotten that because of growth-dissociated product synthesis (*see* page 62), factors which enhance product synthesis also tend to reduce biomass yield or growth rate.

Carbon source

Both the nature and quantity of the applied carbon source have been shown to affect product yield. In particular, increasing sucrose levels tend to improve the level of product synthesis, whereas glucose, although having the same effect on biomass productivity at equimolar concentrations, either does not affect product synthesis or may even be inhibitory (Mantell and Smith, 1983; I. Lyons and M.W. Fowler, unpublished observations). Zenk, El-Shagi and Schulte

(1975) showed that, of a range of carbohydrates tested at the same initial concentration, 2% (w/v) of sucrose gave the highest yield of anthroquinones in *Morinda citrifolia* cultures. The same group (Zenk *et al.*, 1977) also observed enhanced levels of isoquinoline alkaloids in cultures of *Catharanthus roseus* with increasing sucrose concentrations. Increased sucrose levels have also been found to have a positive effect on nicotine biosynthesis (Mantell and Smith, 1983b), diosgenin (Tal and Goldberg, 1983) and polyphenol production (Davies, 1972). However, there are exceptions to this situation: for instance (*see* Mantell and Smith, 1983b) increased sucrose levels did not result in enhanced steroid production in *Solanum ariculare* cultures. The precise nature of the effect of sucrose concentration on product synthesis is not understood.

Macronutrients

This area has recently been reviewed by Dougall (1980). In general, decreasing the key macronutrients such as phosphate, nitrate and ammonia, tends to result in increased product yield. However, this is also generally accompanied by a decrease in growth rate. Dougall and Weyrauch (1980) were able to control anthocyanin accumulation in carrot-cell suspension cultures by using phosphate as the limiting nutrient. Similar observations were made by Wilson (1980) studying anthroquinone biosynthesis in *Galium molugo* cells and Mantell and Smith (1983b) with nicotine from tobacco cells. In a comprehensive study, Knobloch and Berlin (1980, 1981, 1983) working with cultures of *Catharanthus roseus* and *Nicotiana tabacum* have shown that low phosphate in an 'induction' medium aimed at secondary metabolite production, led to substantial increases in tryptamine, indole alkaloids, nicotine and phenolics. Specifically, they observed marked and differential effects of low phosphate on key enzymes of secondary product synthesis. Mantell and Smith (1983b) have suggested that the general effect of low phosphate noted on secondary metabolite biosynthesis may result from depletion of internal phosphate pools which, in turn, results in a lowering of energy charge and derepression of enzymes synthesizing secondary metabolites. The data of Knobloch and Berlin (1983) support this view. Although most observations link low phosphate levels with enhanced secondary product synthesis, there are specific observations which indicate the converse. Two examples are worthy of citation: Zenk, El-Shagi and Schulte (1975) observed increases in anthroquinone synthesis in *Morinda citrifolia*, and Carew and Kreuga (1977) also found increased yields of indole alkaloids in cultures of *Catharanthus roseus*. This latter observation is of particular note as it is diametrically opposed to the observations of Knobloch and Berlin (1983) working with cultures of the same species. Such a stark contrast is difficult to explain, unless other differences in media formulation have an overriding effect.

Although, in general, lowered nitrogen levels tend to enhance secondary product synthesis, no consistent pattern emerges between different nitrogen sources at varying concentrations. A great variety of nitrogen sources have been used in plant-cell culture media, ranging from inorganic sources such as NO_3^- and NH_4^+, to a variety of single amino acids and complex mixtures of the same.

Perhaps the most noticeable effect of varying nitrogen sources on cell cultures is the effect on cell morphology; however, even here there is no consistent pattern.

Growth regulators or phytohormones

This is a wide area with an extensive, if diffuse, literature. Growth regulators are known to have major, often profound, effects on both primary and secondary metabolism of plants. The great majority of cell cultures have an absolute requirement for them, and even where cultures are autotrophic for phytohormones the nature of the requirement is closely defined. As with macronutrients, there is again no truly consistent theme which runs through data from growth regulator experiments. The area of growth regulators has recently been reviewed in Staba (1980) and in Mantell and Smith (1983a) and reference should be made to these for a detailed coverage of the area.

Of all the growth regulators, the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) alone has the general effect of suppressing natural-product synthesis and there are many examples of its action in this way. For instance Zenk, El-Shagi and Schulte (1975), working with *Morinda citrifolia* cultures, found that 2,4-D totally suppressed anthroquinone production. Similar observations have been made with tobacco-cell cultures where 2,4-D appears to suppress nicotine synthesis in a range of cell lines from different varieties of *Nicotiana tabacum* (Furuya, Kojima and Syono, 1971; G.S. Warren and M.W. Fowler, unpublished data).

In contrast to the effect of 2,4-D is the positive effect on secondary metabolite synthesis often seen with other auxins such as indole-3-acetic acid (IAA) or naphthyl acetic acid (NAA) (e.g. Kamimura and Nishikawa, 1976).

The kinins, a group of growth regulators separate from the auxins, appear to have differential effects depending upon the metabolite and origin of the cell line. Constabel, Shyluk and Gamborg (1971) reported that slightly elevated levels of kinetin had a stimulating effect on anthocyanin production in *Scopolia maxima* cultures. In contrast, Shiio and Ohta (1973) found that elevated levels of kinetin totally suppress alkaloid synthesis in *Nicotiana tabacum* cultures.

It has also been argued that cell–cell association and the production of distinctive morphological features may play (together with growth regulator activity) a key part in the development of systems synthesizing secondary metabolites (e.g. Lindsey and Yoeman, 1983). Here again, definitive data which indicate a clear-cut effect on natural-product synthesis are lacking. A number of major problems exist in this area. First, the levels of growth regulators efficacious in plant cells are often at the limits of detection. Secondly, few data are available which indicate the fate of the regulator once it has been applied to the culture. Finally, no credible model of the biochemical action of growth regulators has yet been produced. The singular lack of sound data in this area for predictive modelling is indicative of the unsatisfactory nature of the experimental system. There is an urgent need to achieve a better understanding of the fundamental biochemistry of growth regulator action.

In spite of the lack of fundamental information relating to factors affecting product yield, the adoption during the last few years of empirical microbial

screening techniques has led to some significant enhancement of product yields as illustrated in *Table 5*. It is interesting to note that many calculations for the economic viability of plant-cell cultures are based upon product yields of 1% of the dry weight (*see* for instance Goldstein, Ingle and Lazure, 1980). Several of the products mentioned in *Table 5* have yields of this order, and there are a number of others perhaps only one degree of magnitude away. Given that microbial product yields have in some cases been increased by as much as 2500-fold (e.g. penicillin G), it would be surprising indeed if significant enhancements of plant-product yields were not achievable to at least the level of 10- or 50-fold.

SCALE-UP AND PROCESS DEVELOPMENT

Cell structure and physiology

The large size and slow growth rate of plant cells puts them into a different range of considerations when compared with microbial cells and the problems associated with large-scale growth and process development. Size and growth rate are, of course, not the only points of comparison and difference between plant cells and microbial cells; a more complete list is provided in *Table 6*.

A major facet of plant-cell cultures is the level of cell 'clumping' seen in almost all cultures. Individual cells are only rarely found, instead groups containing from two to as many as 100 cells are typical. Such structures may arise in at least two ways and can be affected by the nature of the cell line, method of subculture, environmental conditions and media composition. In young rapidly dividing cultures they arise predominantly through lack of cell separation following cytokinesis. Occasionally they may also appear in older cultures where excretion of polysaccharide and protein makes the cell surfaces 'sticky' and so cells may adhere together (Fowler, 1982). Various approaches

Table 6. Comparison of the characteristics of microbial and plant cells

Characteristics	Microbial cell	Plant cell
Size	$2\ \mu m^3$	$>10^5\ \mu m^3$
Individual cells	Often	Normally in clumps
Growth of individual cells into colonies	Yes	Not often
Doubling time	>1 hour	Days
Inoculation density	Small	5–20% of total culture
Chromosome number	Haploid/diploid	Mixture haploid, diploid, polyploid, anaploid
Shear stress	Insensitive	Sensitive
Variability within a single culture	Stable	Can vary greatly
Development	Can form spores, pseudo-mycelium	Organogenesis, embryogenesis
Aeration	High, 1–2 vol/vol/min	Low, 0·2–0·3 vol/vol/min
Production formation	Often into medium	Into vacuole

have been attempted to develop 'free' or 'fine' cell suspensions, ranging from alterations in the medium composition, to addition of cell-wall-degrading enzymes (*see* Scragg and Fowler, 1984). None of the approaches has proved ideal, either from the standpoint of maintained culture characteristics or prevention of further clumping. Recently in our own laboratory we have developed a system which will continuously produce fine cell suspensions (Morris and Fowler, 1981; Morris, Smart and Fowler, 1984). However, even when cells are grown on as separate cell lines in liquid suspension, clumping may recur.

The degree of heterogeneity caused by cell clumping makes it extremely difficult, if not impossible, to use turbidometric techniques to assess culture growth. Furthermore, it raises important questions relating to bioreactor configuration and performance and such facets as gas transfer and nutrient availability. None of these points has, as yet, received detailed attention.

The cellulose outer wall of plant cells, although having high tensile strength, makes them susceptible to shear. In consequence there has been a general move towards bioreactors having low shear characteristics (Wagner and Vogelmann, 1977; Martin, 1980; Scragg and Fowler, 1984). This aspect is more fully discussed later. However, a number of cell lines have been grown in conventional stirred tank reactors (CSTR), albeit under conditions markedly different (such as lower turbine speeds) to those for microbial systems. There are indications with some cell lines that a degree of 'internal' cell selection has taken place, giving cells which tolerate relatively high shear levels and retain viability (Scragg and Fowler, 1984).

Not only are plant cells large in comparison with microbial systems, they also have a much lower metabolic activity and, in consequence, a lower oxygen demand. There are comparatively few studies relating the effects of aeration and dissolved oxygen levels to plant-cell growth and productivity. Studies by Kato, Shimizu and Nagai (1975), Smart and Fowler (1981) and by P. Hegarty, N.J. Smart and M.W. Fowler (unpublished results), have shown that metabolic activity, and hence productivity, in plant-cell cultures is particularly susceptible to alterations in the rate and nature of aeration. Recent work in this laboratory has shown that carbon dioxide, as well as oxygen, is a key nutrient in supporting the growth of non-photosynthetic plant-cell cultures (Smart and Fowler, 1981; P. Hegarty, N.J. Smart and M.W. Fowler, unpublished results). However, little is understood of the mechanisms mediating the requirement for carbon dioxide and the precise way in which this affects cellular physiology and biochemistry.

Scale-up

Many of the considerations applicable to scale-up of microbial systems are also applicable to plant-cell cultures. These include such facets as nutrient supply, the nature of the nutrient regimen, uniformity of mixing, culture hold-up, adequate gas transfer, and homogeneity of culture conditions throughout the bioreactor. Little detailed work has so far been carried out on any of these aspects in relation to scale-up of plant-cell cultures. However, there are

reports of the successful growth of plant cells in bioreactors of capacities 0·005–20 m^3, made from a variety of materials and in a range of configurations. Some examples are given in *Table 7*; a more detailed listing is provided in Scragg and Fowler (1984).

Foaming is a problem common to many fermentations, but in the case of plant cells it is particularly acute, with certain cell lines which excrete/secrete large amounts of polysaccharide and/or protein late in batch growth. This causes the cultures to become extremely 'sticky' and the bubbles comprising the foam become encased in a protein/polysaccharide sheath. Cells then become trapped in the foam, gradually forming a crust on top of the culture. In addition, cells stick to solid/liquid interfaces, particularly around probes and entrances to ports into and out of the bioreactor. Little is understood of the nature of this foam system and no procedures have yet been devised which provide total relief from it. A number of workers have attempted to use antifoam reagents, with varying degrees of success (*see* Martin, 1980; Smart, 1984: also Chapter 10 of this volume).

Table 7. Examples of large-scale culture systems for plant cells

Year	Volume (litre)	Vessel type	Plant species	Reference
1959	30; 134	Stainless steel tank, bubble column	*Gingko, Lolium*	Routier and Nickell, 1956
1963	7·5; 15	CSTR	*Daucus carota*	Byrne and Koch, 1962
1975	65–1500	Stainless steel and glass bubble columns	*Nicotiana tabacum*	Kato *et al.*, 1976
1977	15500	Stainless steel CSTR	*Nicotiana tabacum*	Noguchi *et al.*, 1977
1981	100	Glass air-lift loop	*Catharanthus roseus*	Smart and Fowler, 1984a

Bioreactor configuration and performance

The earliest recorded work on large-scale growth of plant cells comes from the mid-1950s. Much of the work at this time was carried out in what were little more than converted carboys, in some cases using impellar mixing, in others a bubble column (for review *see* Martin, 1980). Although none of this early work led to a process system, it did demonstrate the feasibility of growing plant cells on a fairly large scale (up to about 0·2 m^3), particularly in relation to maintaining sterility and cell viability for long periods. It was, perhaps, the move to commercially available CSTR systems that had been specifically designed for micro-organisms, which led to an appreciation of the key differences between plant cells and micro-organisms (Byrne and Koch, 1962; Kaul and Staba, 1967). Nevertheless, workers at the Japanese Salt and Tobacco Monopoly successfully grew tobacco in a CSTR of 20 m^3 (Noguchi *et al.*, 1977). However, during the 1970s it became apparent that, although it was possible to grow some lines of plant cells in CSTR systems, they were the

exception rather than the rule. The alternative approach of using air-driven reactors, where the gas stream serves not only to aerate but also to mix the culture, has subsequently found favour in a number of laboratories (*see* Martin, 1980; Fowler, 1982; Scragg and Fowler, 1984).

Air-driven reactors in general have a much lower shear characteristic than CSTR systems and, in consequence, a much higher viability is achieved with plant cells. A further feature is also of importance in relation to plant-cell cultures: plant cells have a much lower oxygen demand than microbial cells (the maximum requirement for plant cells is about 1·8 mmol O_2/ℓ/hour compared with 10–15 mmol O_2/ℓ/hour for yeasts and from 5–90 mmol O_2/ℓ/hour for bacteria). The situation consequently resolves into the need to achieve good mixing of nutrient and biomass, relative to low shear and a low oxygen demand.

Only one detailed analysis has so far been carried out relating bioreactor design to culture performance with plant cells. The study was carried out by Wagner and Vogelmann (1977) with cultures of *Morinda citrifolia* as the test system. Comparisons were made of four bioreactor configurations: a CSTR with a flat blade impellar; a stirred tank with a perforated disc impellar; a draught-tube bioreactor with a Kaplan turbine, and an air-lift bioreactor with a draught tube. Although the final dry-weight values of biomass achieved were similar in all cases, the production of anthroquinones was greatest in the air-lift draught-tube system. In recent years both major types of air-lift reactor, that is the draught-tube and the loop system, have been used successfully for the large-scale growth of plant cells. Zenk *et al.* (1977) used a 0·03 m^3 draught-tube system with cultures of *Catharanthus roseus* and recorded both good culture growth and high yields of alkaloid. Similar results have also been achieved with *C. roseus* cultures grown in air-lift loop reactors of between 0·01 and 0·1 m^3 capacity (Fowler, 1981; Smart and Fowler, 1984a,b).

While agreeing with the general concept that air-driven bioreactors may be the most appropriate for plant-cell growth, recent studies by Tanaka (1981) suggest that at biomass densities above about 20 g/ℓ dry weight, certain restrictions begin to appear. In particular, he observed that at high biomass levels, air bubbles did not separate from the culture. In consequence, circulation became impeded and unstirred regions developed, giving a multiphasic system. Similar observations have also been made by Wilson (1978). There is undoubtedly a need for a thorough study of bioreactor configuration and performance in relation to culture characteristics, to allow an appropriate choice of large-scale growth system.

Process systems

A relatively detailed literature is now available recording the growth of plant cells in batch, semi-continuous (fed-batch) and continuous culture. Batch culture is most often used for plant-cell culture. Detailed information on this can be found in Street (1977) and in Thorpe (1978).

Semi-continuous culture has been little investigated, except for some work by Kato *et al.* (1977) with tobacco cells and, recently, a more comprehensive study by Dougall's groups (Dougall, LaBrake and Whitten, 1983a,b). Dougall

and co-workers demonstrated very clearly that the factors which affect biomass productivity often have no effect on specific product synthesis; where they do, it is typically an adverse one. A key facet of this is the 'uncoupling' between biomass and secondary metabolite synthesis. The fact that this appears to occur in the majority of situations where the synthesis of a particular secondary metabolite is desired, may be turned to advantage using a semi-continuous system. By closely following the production of biomass and the desired product (*Figure 4*) it should be feasible to select an optimum time point when the cells are still dividing, albeit slowly, in late exponential phase and when there is a reasonable level of product present. At this point the major part of the culture (biomass and nutrient) is harvested and fresh nutrient added to make up the volume to the original level. In this way a culture may be maintained operational and productive for an extended period; furthermore, equipment and process time are used efficiently. We have taken cultures of *Catharanthus roseus* through three or four cycles of growth and nutrient change before the culture has eventually failed (D. Grey and M.W. Fowler, unpublished results). At present we do not know the reason for the sudden decline in cell viability which occurs after the third or fourth change. One possibility is that toxic by-products build up in the nutrient, carried over to some extent between each nutrient loading, and eventually promote cell death. Semi-continuous culture does have a number of advantages as a process system and is most certainly worthy of further exploration.

Continuous culture of plant cells has been explored to varying degrees (for reviews *see* Fowler, 1977; Wilson, 1980). Much of the early work was concerned more with assessment of whether or not plant cells grown in such systems obeyed classic Michaelis–Menten kinetics, as derived for microbial cultures, than with assessment of continuous culture as a potential process system. Initial indications were that plant-cell continuous cultures obeyed

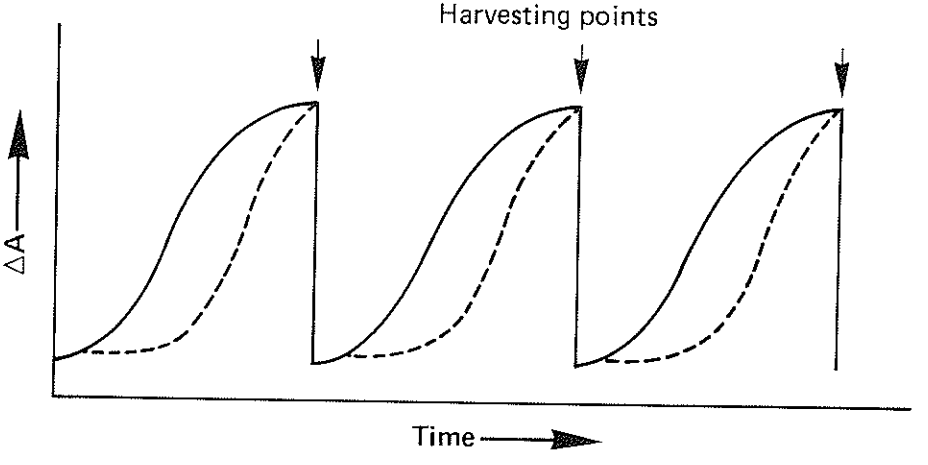


Figure 4. Semi-continuous mode of operation for natural-product synthesis. ——— Biomass; – – – natural product; ΔA, change in biomass or specific product level.

Michaelis–Menten kinetic models (Wilson, King and Street, 1971). However, more detailed studies by Dougall and Weyrauch (1980) and Sahai and Shuler (1982) indicated that this was not universally the case, and that deviations occurred and were particularly affected by such features as wall growth, bad mixing and the consequent development of heterogeneous zones in the bioreactor. Such deviations are not just of academic interest, but have important implications for attempts at scale-up predictions for mass cell-culture operations.

Although continuous culture has a number of obvious advantages as a process system, its potential is restricted because of the lack of a positive relationship between biomass production and product synthesis. In consequence, continuous culture is probably not going to be a major process system for natural-product synthesis from plant cells. A good illustration of the problem comes from the recent work of Tal and Goldberg (1982) who studied steroid production by cells of *Dioscorea deltoidea* in a 0·002 m^3 fermenter under both batch and continuous culture modes. In continuous culture good cell growth was achieved, but little steroid synthesis. In contrast, batch cultures gave good cell growth and steroid production, the latter occurring principally as cells entered stationary phase.

Although continuous culture is probably not viable as a sole mechanism for natural-product synthesis from plant cells, it may nevertheless have a role as the first part of a two-stage system. In this mode the continuous culture first stage would provide a continuous and rapid supply of biomass. The second stage, of either a batch or immobilized-cell system, would then be adjusted to give product synthesis. This approach has already been used, firstly in the development of the β-methyl digitoxin biotransformation process (Reinhard and Alfermann, 1980) and secondly in the Mitsui process for shikonin production (Curtin, 1983).

Little mention has so far been made of immobilized-cell technology. This has undergone rapid development in recent years and a great deal of work has been carried out with plant cells (for reviews *see* Lindsey and Yoeman, 1983; Rosevere and Lambe, 1984). Unfortunately, such a system relies upon the desired product being released into the bathing medium, from which it is then recovered. In the case of plant cells, secondary metabolites are typically accumulated in the cell vacuole, from which it is extremely difficult to effect their release without either killing the cells or markedly reducing productivity. Until some way can be found of achieving this, immobilized systems have a limited application.

The future

With the advent of the Mitsui Petrochemical Industry processes for shikonin and berberine, and the rapid development of processes for cardiac glycoside and alkaloid production, plant-cell culture can be said to have 'come of age'. However there is still much to be learned about the nature of the cell system, its physiology and biochemistry and, above all, its plasticity in responding to the various selection pressures applied to enhance biomass and product yield.

While the next decade will undoubtedly see a number of other plant-cell processes come to fruition, much fundamental scientific research needs yet to be done before a more widespread application is feasible.

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