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REVIEW

Biotechnological Applications of Plant Cells

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Introduction

The ability readily to culture various plant tissues and organs has been utilized by plant scientists for a number of years. Reinert and Bojaj (*1*) adequately detail in their book the techniques to be used and the advantages to be obtained from the cultivation of organs and meristems from a variety of plant species for commercially important reasons. Such reasons include the propagation and improvement of plant species, the establishment of virus-free lines of plants, and the creation of new species by genetic manipulations.

The substance of this review deals solely with biotechnological applications of plant cell suspension cultures. It is this type of culture that is capable of being exploited, and indeed is being exploited as a replacement for whole plants in their classical role as providers of industrially important pharmaceutical products. The potential advantages to be gained by using plant cell cultures include:

(A) Growth of large amounts of plant tissue in minimal amounts of space.

(B) Ability to control the growth conditions and nutrients received by the cells.

(C) The growth of cells is carried out under sterile conditions.

(D) Ease of harvesting the cells, and of extracting the desired product(s) (which may even be excreted into the medium).

(E) Compared with whole plants the investigator is essentially dealing with a homogeneous population of cells.

Conditions for the Growth of Plant Cell Suspension Products

To obtain a suspension culture of plant cells, one must first produce a callus culture. A callus, which consists of masses of unorganised tissue, is obtained by placing a freshly cut sterile section of young vigorously growing plant tissue upon

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the surface of agar gel medium. Once a suitable callus culture has been established, then portions of this can be shaken in a suitable liquid medium, to create a cell suspension culture (*2, 3*). Most such suspension cultures appear to consist of small groups of cells varying in number from a few to many (*4*).

Defined media for the growth of plant cells in suspension culture, consist of mineral salts, a carbon source, and plant growth regulators. Although many such media exist, a few basic types are in general use. The B5 medium of Gamborg et al. (*5*), Murashige and Skoog (MS) medium (*6*), and Eriksson's (ER) medium (*7*) are probably the most widely used.

Studies of a pilot nature can be carried out with suspension cultures shaken in flasks of up to 500 mL capacity. To grow plant cells on a large scale, however, the research worker will presumably be interested in growing batches of the cells under fermenter-type conditions. Examples of fermenter machines can be seen in refs. (*8, 9*). The fermeter designed by Kurz (*8*) uses compressed air for agitation and has the advantage that it encourages cultures to grow as single and double cells. This will increase the cell surface contact with medium, when compared with the aggregates normally obtained from other culture methods. The Kurz fermenter has been used for the continuous culture of a number of different cell lines for periods of up to three months, in B5 media. Under these conditions the dry weight of a soybean

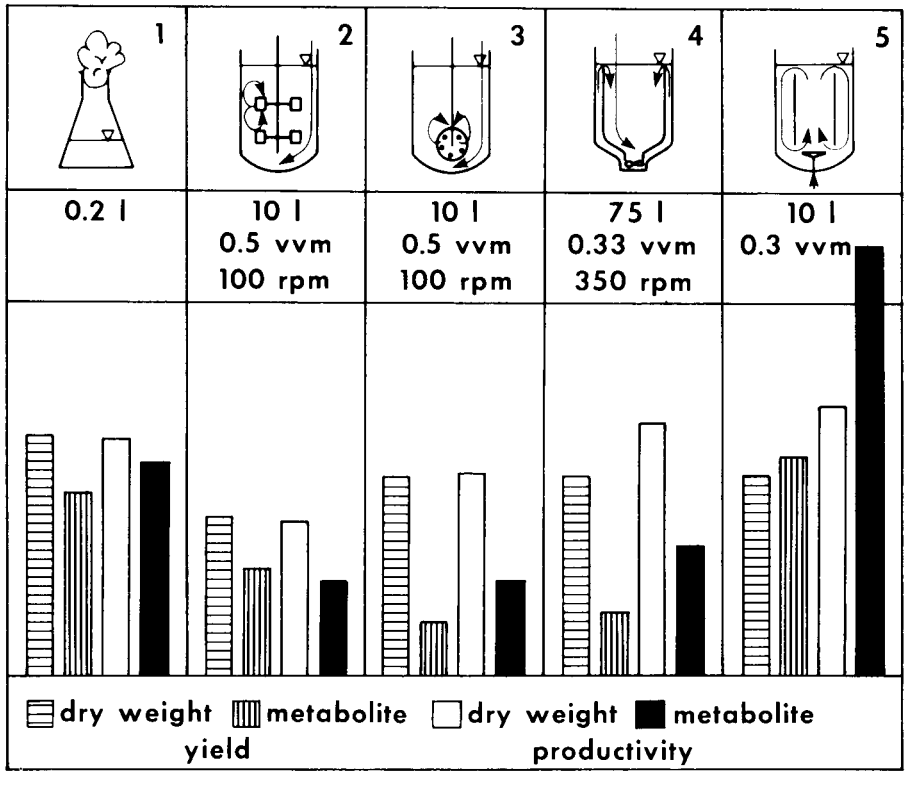

Fig. 1. Comparison of yield and productivity for cell mass and anthraquinones in various reactor systems: 1, shake flask; 2, flat blade turbine; 3, perforated disk impeller; 4, draft tube reactor with kaplan turbine; 5, airlift reactor (from Zenk et al., *29*).

cell culture grown with a generation time of 30 h, equilibrated at 1.3 mg/mL of culture (*10*).

Theoretical and practical aspects of plant cell growth in bioreactors are dealt with in ref. (*9*). Wagner and Vogelman discuss the productivity of the plant species *Morinda citrifolia* when grown in bioreactors of different designs. They show that the yield of cells and the anthoquinone products varies significantly depending upon reactor design. Thus the so-called airlift reactor that again uses compressed air for aeration gives yields of anthoquinone that are almost double those of other reactor designs (Fig. 1). The increased productivity in this case is explained by low cell shear rates, and sufficient oxygen supply, when compared with growth in bioreactors using mechanical impellers for mixing.

The growth of plant cells with continuous addition of medium, and removal of a balancing amount of culture, can be designed to maintain the system at a constant submaximal growth rate. This type of system is termed open continuous culture (*11*). In this article Fowler discusses the theoretical background kinetics for such a system, and in particular the chemostat situation, where the concentration of a single nutrient has been adjusted so as to be growth limiting. Such a situation is of potential use in studying the nature of any particular nutrient on productivity, and also the effect of structurally or functionally related nutrients.

Other authors such as Street (*12*) and Wilson (*13*) have also reviewed types of apparatus available for the large scale growth of plant cells with their attendent advantages and disadvantages.

The Potential for the Production of Secondary Metabolites by Plant Cells

Plant cells growing in culture are regarded as being totipotent, since cell cultures of a number of different plant species can be induced to regenerate whole plants (*14, 15*). Since cells growing in culture have been found to be totipotent in a morphological sense, they are usually considered to be totipotent in other senses, such as the production of secondary metabolites (*16*). In a number of instances, cell cultures have apparently not lived up to the expectations of either the morphologists or the chemists. Although many plant species do not respond to attempts to initiate organogenesis (*14*), many earlier attempts at the production of secondary products using cell culture methods also failed (*16*). One possible reason for these failures was considered to be loss of totipotency by alteration of the characteristic plant genome. It should first of all be acknowledged and appreciated that plant cells grown in culture may not have stable chromosome numbers (*17–19*). In spite of this fact, genome alteration does not seem at present to be considered the probable cause of an apparent lack of totipotency in any sense of the word. Thus as more knowledge becomes available about organogenesis, more species of plants are shown capable of regeneration from culture, and plant cell cultures have become available that will produce secondary products in as good or better yields than whole plants.

Street (*20*) emphasizes the fact that the various selection pressures existing in plant cell culture conditions are likely to lead to cytological changes, and the production of new lines of cells. Such pressures include changing from one culture system to another, from batch to continuous culture, for example. For this reason, producing strains of plant cells should be checked during all phases of their culture in order to prevent total loss of a producing strain (*21*).

The changes that may be seen in the production of secondary metabolites by a variety of different cell lines are considered to be most often caused by epigenetic changes involving not gene loss, but the way that genes are expressed. Probably the best evidence for epigenetic changes being associated with plant cell cultures comes from work carried out on habituation of plant cells. Cell cultures of tobacco tissue for example, occasionally lose their requirement for either auxins, or cytokinins, or both (*22, 23*). This process is known as habituation, and such tissues appear to have altered abilities to control their growth; for example, tumors are formed when the cultured tissues are grafted onto the normal host (*24*). It is, however, possible to regenerate complete plants from cloned lines of tobacco tissue, habituated for both cytokinins and auxin (*25*). Thus habituation is assumed to be an epigenetic change, not an alteration of totipotency.

Optimization of Secondary Product Production by Plant Cell Cultures

Investigations have been carried out to determine the effect of a large number of parameters on the growth of cells and their production of secondray metabolites. Among these parameters are hormone and growth factor levels, light intensity and quality, nutrient constituents of the growth medium, gas phase, and temperature.

One of the most extensive experiments designed to determine the effect of varying a number of the above parameters was that of de-Fossard et al. (*26*). This experiment utilized 81 different media, generated by varying the concentrations of minerals, auxins, cytokinins, and sucrose plus growth factors plus amino acids. The results were studied in terms of growth and development of the cultures, but not in terms of secondry product formation.

The work of Brain (*27*) showed the importance of some of the same factors studied by de-Fossard et al. in the production of L-dihydroxyphenylalanine (L-DOPA) by *Mucuna pruriens* cell cultures. Most investigators would utilize 2,4-dichloroacetic acid (2,4-D) levels in the range of 0.1–5 mg/L for the growth of cell cultures. Brain found that 25 mg/L of 2,4-D led to optimal production of L-DOPA, which was released from the cells and accumulated in the culture medium in the amount of 1% w/v of the medium. This finding is of obvious practical value, in view of the use of L-DOPA for the treatment of Parkinson's disease.

The production of the antitumour alkaloid camptothecin, by cell cultures of *Camptotheca acuminata,* was investigated by Misawa et al. (*28*). Cells were found to grow best with optimal levels of 2,4-D, kinetin, and Gibberellin A3. Sucrose was found to be the preferred carbon source. Various amino acids were utilized as possible precursors of the alkaloid. It was found that 0.115 m*M* L-tryptophan or

L-phenylalanine stimulated growth. In spite of the stimulatory effect of L-tryptophan on growth and the fact that it is considered to be a precursor of campothecin, no increase in alkaloid content of the cells was observed. The final yield of camptothecin obtained was $2.54 \times 10^{-4}\%$ of dry weight compared with $5 \times 10^{-3}\%$ of dry matter for the whole plant.

Zenk et al. (*29*) have carried out detailed studies demonstrating methods to be utilized in obtaining plant cell lines that produce Rauwolfia alkaloids. Ajmalicine, important in the treatment of high blood pressure, has been shown to be produced by approximately 28 species of plants (*29*). It can also be prepared from the alkaloid serpentine, by reduction with BH_4. Zenk et al. started with the goal of comparing the alkaloid contents of cell cultures with the plants of the particular species they were obtained from. To do this, they first selected whole plants of the species *Catharanthus roseus*, which had a wide spectrum of alkaloid contents, ranging from 0.93% to 0.17%. To accurately estimate the ajmalicine and serpentine contents of these plants, they utilized sensitive radioimmunoassay (RIA) techniques that enabled them to screen many samples of plant material with high accuracy. Cell suspension cultures were developed from seedlings grown from seeds of selected plants, and a comparison made of the alkaloid yields of cultures, and plants from which the cultures and plants were derived. Zenk et al. found that the production of alkaloids by cultures derived from high yielding parents was considerably higher than those derived from low yielding parents. By contrast, the average cell yield was reasonably comparable in both cases.

Further experiments were carried out to improve the yields of alkaloids obtained from the high yielding cell lines. The RIA screening procedure was used together with a filtration method to obtain single cells that were cultured on an alkaloid production medium containing 0.8% agar. Additional work showed that alkaloid production was strongly influenced by the composition of the medium. Most significant were results showing that while indol-3-acetic acid (IAA) gave high cell and alkaloid yields, 2,4-D and naphthalene acetic acid (NAA) suppressed alkaloid formation. Benzyladenine in the absence of an auxin source gave high yields of alkaloid, but low cell yields.

Studies of the influence of various possible precursors of the alkaloids showed that no marked stimulation of any kind was obtained with any of the shikimic acid pathway intermediates. A threefold stimulation of alkaloid biosynthesis was obtained with L-tryptophan itself, however. Also interesting was the fact that synthesis was not positively influenced by substituted tryptophan derivatives. This is unlike the fungal (Claviceps) system, where indole alkaloid production can be positively influenced by metabolites such as 5 or 6-methyltryptophan (*30*).

Utilizing stable, high-producing cell lines plus their alkaloid production medium, Zenk et al. (*29*) were able to obtain combined concentrations of serpentine plus ajmalicine, equal to 1.3% of the total cell dry weight. This value exceeds that found in the original whole plants by a factor of 1.5, and from the intact root tissue by a factor of 5. The total scheme utilized by Zenk et al. is shown in Fig. 2.

Other workers have also shown the importance of selection and screening programs in obtaining high-yielding plant cell lines. Tabata et al. (*31*) have described cloning and cell squash procedures used in obtaining a tobacco cell line able to pro-

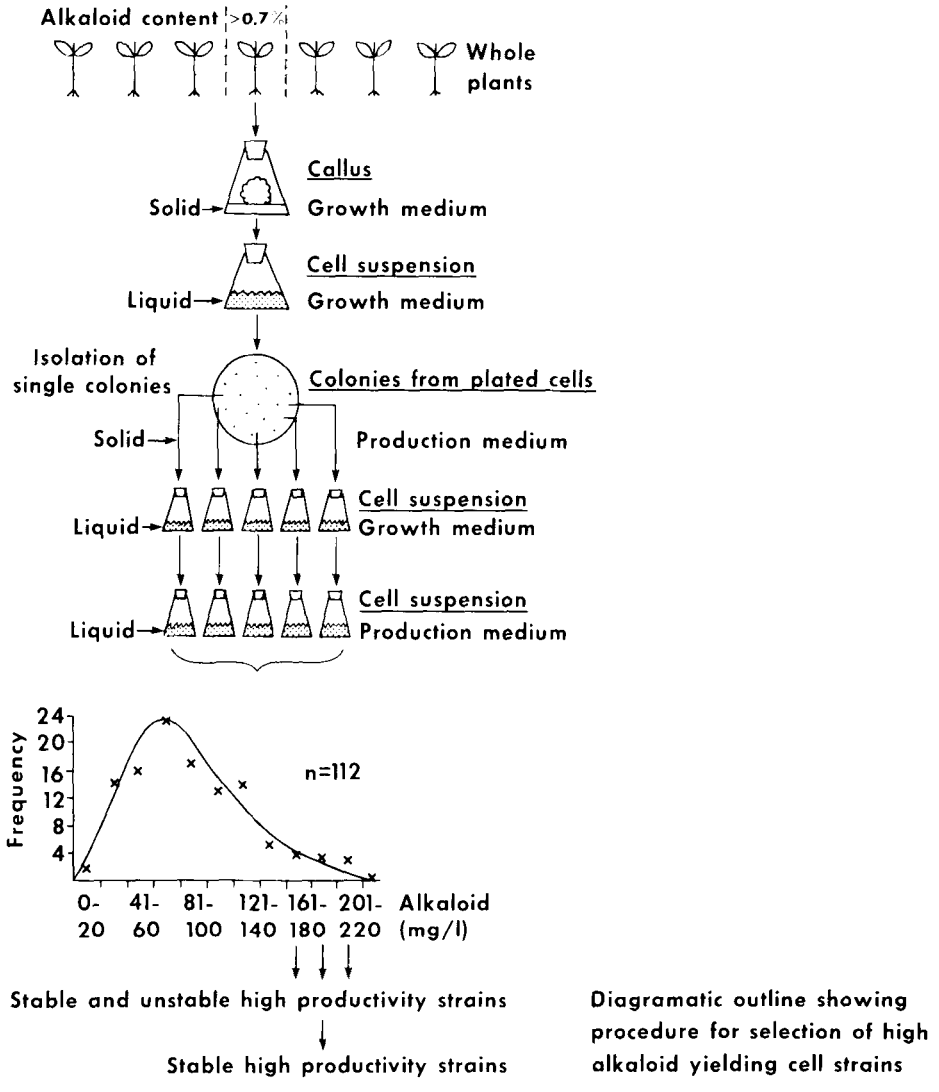

Fig. 2. Diagramatic outline showing procedure for selection of high alkaloid-yielding cell strains (from Zenk et al., *29*).

duce nicotine in up to 2–5% dry weight, comparable to the highest yields obtained from tobacco plants.

Many other examples of cultures are now known that yield remarkably high quantities of natural products. Cultures of *Panax ginseng* produce ginsenosides up to 21% of the dry weight as crude saponins (*32*). Cell suspension cultures of *Dioscorea deltoidea* produce diosgenin, the major raw material in the commercial production of corticosteroids of various kinds, up to 1.5% of the dry weight (*33*).

Lithiospermium erythrorhizon cultures have been utilized successfully in attempts to increase the content of naphthoquinone pigments relative to whole plant tissues. Tabata et al. (*34*) have managed to obtain pigment contents of 12% of the dry weight of the cell suspensions, which is about 8 times that of the root tissues

used as a source of the drugs. In Japan and China, these pigments are used for the the treatment of burns and various skin ailments (*35*).

Biotransformation by Plant Cell Cultures

The biotransformation of steroids by a large variety of different plant cultures has been extensively studied (*36*). Experiments using pregnenolone and progesterone have outlined the most common types of transfomration seen in plant cell culture. These involve reduction of 3-keto and 20-keto groups, as well as $\Delta^{4,5}$ double bonds with the subsequent formation of glucosides or palmitates (*36*). Furuya et al. (*36*) has also examined the metabolism of a variety of 5β-pregnane derivatives. Results have shown that a variety of products can be isolated, these indicate that in general the metabolism of 5β-pregnane derivatives is similar to that of 5α-pregnane derivatives, yielding hydroxylated derivatives and their glycosides.

Both Stohs (*37, 38*) and Furuya et al. (*36*) have also investigated the metabolism of androstane derivatives. Furuya found that cell cultures of *Nicotiana tabacum* were capable of carrying out a variety of transformation reactions using testosterone as the starting material. Particularly interesting was the formation of 5α-androstane-17β-ol-3-one, which has been recognized as an active form of testosterone in animals.

The ability of *Digitalis lanata* cell cultures to carry out reactions leading to the eventual formation of cardiac glycosides has been demonstrated by Reinhard et al. (*39*). These investigators were particularly interested in the ability of cultures to carry out the hydroxylation of the digitoxin nucleus to digoxin, which is of significant pharmaceutical value. They were able to obtain approximately 7% conversion rates of β-methyl digitoxin to β-methyl digoxin, using an airlift fermenter, and 12-day incubation periods (*40*). Heins (*40*) has also extensively utilized screening methods, designed to find cell strains with high and selective 12β-hydroxylation capacity. These studies enabled them to conclude that the hydroxylation capacities of tissue cultures could not be correlated with the digoxin contents of the whole plants they were obtained from. Instead, it was suggested that when searching for sources of plant tissue to initiate cultures, it is more important to screen plants for enzymatic hydroxylation capacity.

The biotransformation of terpenoids by plant cell cultures is also of interest. The diterpenoid stevioside is widely used as a natural sweetening agent (*36*). Furuya et al. (*36*) have investigated the biosynthesis of this and other derivatives with a sweet taste in cultures of *Steria rebandia* and *Digitalis purpurea*.

Although the biotransformation of alkaloids has not been as extensively studied as that of other groups of compounds, some useful work has been carried out in this area. Thus Wink et al. (*41*) have shown that cadaverine can be incorporated into the quinolizidine alkaloids by cell cultures of *Lupinus polyphyllus*. Identified transformation products included sparteine, 17-oxosparteine, and lupanine. These studies have been useful in providing confirmatory evidence for the alkaloid biosynthetic pathways thought to exist in this plant.

Production of Enzymes by Cell Cultures

In contrast to the wide use made of various microorganisms as industrial sources of enzymes, studies of plant cell cultures have not focused on their abilities to synthesize enzymes and other proteins for industry. In studying the production of secondary products by plant cells, however, a beginning has been accomplished in two areas of potential importance to the eventual production of enzymes on a commercial basis. First, the investigation of factors involved in the regulation of enzymes in plant cell cultures, and the isolation of specific enzyme systems. Second, a serious effort has been made to obtain reliable auxotrophic mutants of plant cells. This later work will be dealt with in the following section dealing with manipulations of the plant cell genome.

Although many pathways such as those utilized in amino acid biosynthesis, for example, are similar in bacteria and plants (*42*), the regulation of these pathways differs in at least one important aspect. Induction and repression as thought to operate in microorganisms by Jacob and Monod (*43*), for example, do not appear to play a major role in the regulation of enzyme synthesis in plant cells. Enzymes in plant cells are, however, subject to synthesis and degradation (turnover), and a recent book on this topic discusses the subject in some detail (*44*).

Probably some of the clearest work carried out on the induction and synthesis of enzymes in plant cell cultures, has been concerned with the biosynthesis of flavonoids. Hahlbrock et al. (*45, 46*) have looked extensively at the role of light and other factors in the regulation involved in flavonoid biosynthesis in parsely cell cultures (*Petroselinium hortense*). Using these cultures, Hahlbrock et al. found tha the synthesis of enzymes of the lignin and flavonoid biosynthetic pathways could be specifically induced by UV radiation in the 320–350 nm range. Hahlbrock et al. also found that the nine enzymes involved in the biosynthesis of the flavonoid glycoside, malonyl apiin, from phenylalanine, reached peak activities 20–40 h after exposing parsley cells in stationary phase to UV light. In addition, three of the enzymes involved in general phenyl propanoid metabolism—phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase, and *p*-coumarate:CoA ligase—could be induced by stimuli other than light, e.g., dilution of cultures into distilled water or fresh medium.

The Hahlbrock group have also carried out detailed studies on the turnover of PAL in parsley cell cultures (*47–50*). These studies led them to the conclusion that observed changes in the rate of enzyme synthesis arose from corresponding changes in the amount of translatable PAL-mRNA. Thus it would seem that in this case a true inductive effect is produced by the action of UV light on parsley cells. Hahlbrock and various other workers (*51–56*) have also worked on the purification and properties of enzymes other than PAL that are involved in flavonoid biosynthesis.

Work of potential biotechnological importance is also being carried out on enzymes involved in the synthesis of medically important alkaloids, vincristine and vinblastine. These alkaloids have significant importance in the treatment of Hodgkin's disease and other forms of cancer (*57*). Efforts to obtain the synthesis of these alkaloids in cell cultures of *Catharanthus roseus* have so far been

unsuccessful (*58*), and large amounts of mature plant tissue have to be processed to obtain minute quantities of the drugs. Madyastha and Cosca in an extensive review article (*59*) describe work carried out on the partial purification and the study of enzymes of the pathway leading to vincristine and vinblastine. In addition to the isolation of a number of enzymes involved in the biosynthesis of secologanin from monoterpene precursors, enzymes involved in the biosynthesis of strictosidine and ajmalicine from tryptamine and secologanine have either been isolated or detected in *Catharanthus roseus* cell cultures.

The purification of these and other enzymes involved in the synthesis of mono- and bis-indole alkaloids from plant cells could conceivably facilitate the synthesis of important anticarcinogenic drugs under in vitro conditions.

Various enzymes isolated from plant sources have already been utilized and shown to be of potential importance for biotechnological purposes; thus, for example, immobilized barley α-amylase has been utilized for the hydrolysis of starch (*60*). Papain (*61*), ribulose bis-phosphate carboxylase (*62*), and pectin esterase (*63*) have been immobilized on porous glass beads. Soybean trypsin inhibitor has been crosslinked and used for the separation of trypsin and peroxidase (*64*). Papain has been immobilized on chitin and used to remove haze in beer (*65*). It would appear, therefore, that what is needed is the co-utilization of existing knowledge in plant tissue culture techniques on the one hand and enzyme technology on the other hand. This should hopefully bring about the development of the apparently untapped sources of enzymes that can be provided by plant cell cultures.

Methods Used for the Production of Genetically Different Types of Plant Cells

The production of genetically different plant cells growing in tissue cultures may be accomplished by a variety of methods that fall under one of the following headings:

1. Protoplast fusion.
2. Alteration of the genome characteristic of individual cells.

Protoplast fusion has achieved prominence as a technique that may eventually be used by plant breeders, as a powerful adjunct to traditional breeding methods (*66*). Using this technique, protoplasts of two entirely different cell lines may be fused, usually with the aid of polyethylene glycol (*67*). If the fusion of protoplasts is followed by a subsequent fusion of nuclei and successful genome and cell divisions, the subsequent growth and manipulation of the hybrid obtained may yield a new plant species. Presumably, an informed choice of parental strains of cell lines for this fusion process could be of significance in increasing the yield of a secondary product obtained in cell culture. At the present time, however, the protoplast fusion technique appears to be developing relatively slowly and has not as yet lent itself to quantitative work of this type.

The alteration of the genome of an individual cell may be brought about by either mutation at one or more loci in the existing chromosomes, or by the introduction of foreign DNA into the nucleus of the cell.

Over the last 10–20 years, interest in the production of mutants has significantly increased in line with the interest in plant tissue cultures. Two recent review articles (*68, 69*) attest to the significant volume of work being carried out in this area. To be of use in biotechnology, mutant plant cells would obviously have to differ significantly from the normal cells that are already available. Such differences might involve the overproduction of a particular product of interest, such as a normal metabolite, or the synthesis of an entirely new product.

Many mutants are now available that overproduce certain metabolites, the majority fall into the group termed resistance mutants. The isolation of plant cell mutants is usually (but not always) preceded by the treatment of plant cells with a suitable mutagen. Chemical mutagens such as ethylmethanesulfonate (EMS), nitrosoguanidine, or nitrosourea are most often used, and lead to an increase of about 10-fold in the frequency of isolation of mutant cell types (*68*).

Some kind of selection technique may be applied after the use of a particular mutagenic treatment to select cells with resistance to a particular drug. Direct selection utilizes the ability of a mutant cell line to grow under a set of growth conditions that are deleterious to the wild type or normal cell. Such conditions usually consist of growth in the presence of an antibiotic such as streptomycin (*70*), herbicides such as 2,4-D (*71*) or picloram (*72*), various amino acid analogs (*68, 69*), or an antimetabolite such as aminopterin (*73*). Usually the selective conditions are first defined by testing the effect of various drug concentrations on growth and survival of cells in liquid or agar culture. Subsequently, large populations are screened for the presence of cells able to divide in the presence of a toxic drug, the amount of the drug used over the course of a number of growth cycles is gradually increased.

When the nature of resistance mutants is examined, they are frequently found to have acquired a permanent hereditable change in the genotype and phenotype (*72–74*). This is not always the case however, and Maliga (*68*) points out that in order to definitely distinguish phenotypic changes arising from permanent mutational events from those caused by altered gene expression, Mendelian analysis is requried. For this to be carried out, sexual expression of genes in regenerated plants is required. Workers in this area should also be aware that resistance to some drugs, in particular streptomycin, most likely involves a change in the DNA of the plastids, as indicated by the maternal inheritance of the trait (*70*).

A number of cell lines that overproduce various metabolites are now known. The various 5-methyltryptophan (5-MT) resistant cell lines developed by Widholm et al. (*69*) all accumulate more than 20 times the normal amount of tryptophan. In most of the cell lines, this is apparently the result of the possession of an altered anthranylate synthetase enzyme, less sensitive to feedback by 5MT, and tryptophan. A selection of other resistant lines that overproduce amino acids of various kinds are shown in Table 1.

A few of the amino acid overproducing cell lines are of special interest in that they also overproduce secondary products derived from the amino acids. Carrot

Table 1
Some Mutant Plant Cell Strains That Overproduce Certain Amino Acids

Reference	Plant cells used	Drug used	Amino acid overproduced
79	*O. sativa*	Aminoethyl-cysteine	Lysine
80	*D. carota*	Aminoethyl-cysteine	Lysine
	D. carota	α-Amino-caprylic acid	Lysine
	D. carota	Selenomethionine	Lysine
81	*D. carota*	Hydroxyproline	Proline
	D. carota	Ethionine	Methionine
81	*N. tabacum*	δ-Hydroxy-lysine	Lysine
	N. tabacum	Aminoethyl-cysteine	

and tobacco cell lines that oversynthesize phenylalanine were selected from unmutagenized cell lines grown in media containing inhibitory levels of *p*-fluorophenylalanine (PFP) for 2 months. The tobacco cell line is apparently synthesizing more phenylalanine than normal because of an altered chorismate mutase enzyme that is less sensitive than normal to feedback inhibition by phenylalanine, tyrosine, and PFP (*76*). In this line phenylalanine does not accumulate, but is converted into phenolics that do accumulate. Berlin and Widholm (*69*) have established that phenylalanine ammonia lysase (PAL) levels are about ten times higher than normal in the PFP-resistant cells, this presumably indicates that the regulation of phenyl propanoid metabolism is also affected. The mutant carrot cell line, by contrast, accumulates phenylalanine and not phenolics (*76*). Both resistant lines show a markedly reduced uptake and incorporation of PFP and phenylalanine.

A carrot cell line resistant to 5-MT can overproduce tryptophan to give a 25% increase in total cell tryptophan content (*77*). Similar carrot lines also overproduce IAA, rendering them auxin autotrophic (*68, 78*).

Rather more specific selection methods of various types have been utilized in an attempt to isolate auxotrophic mutants. Probably the best example of such a technique, one that has proven successful with plant cells, is growth in chlorate-containing medium, designed as a means of isolating cells deficient in the enzyme nitrate reductase. Cells that possess nitrate reductase will be selected against, since they reduce chlorate to the toxic metabolite chlorite (*82*). King and Khanna (*83*) have isolated a number of such mutants with varying properties and sensitivities to chlorate.

Selection methods based on the use of nucleic acid analogs and antimetabolites have found wide use in the study of mammalian mutants. The hypoxanthine, aminopterin, thymidine, or HAT medium [used for selection of mutants in the sal-

vage pathways of pyrimidine and purine metabolism (*84*)] is probably one of the best known systems of this type. Attempts have been made to isolate 5-bromodeoxyuridine (BUdR) and 8-azaguanine (AG) resistant plant cells (*68*). These two analogs have been utilized with mammalian cells to select mutants that are thymidine kinase (TK), or hypoxanthine guanine phosphoribosyl transferase (HPGRT) deficient (*84*). The use of HAT medium can detect recombinants between such cells, which must possess both functioning purine and pyrimidine salvage pathways in order to survive. Selection of plant cells with reduced HPGRT activities has been achieved (*85*) after use of AG. All BUdR isolates, however, have exhibited significant TK activity (*86–88*). The latter result may possibly be explained by the existence of two TK enzymes in plants (*89*). In this case, inactivation of two genetic loci would presumably be necessary to obtain no TK activity.

Many other workers have also had problems with leaky mutants. In theory, this problem should be overcome where only one locus is present per cell. Carlson (*90*) used haploid cell cultures of tobacco to derive a series of auxotrophic mutants. Cultures of cells (65–80% haploid) were exposed to EMS, and then to selection via the BUdR suicide technique. In this method, only auxotrophic cells that do not grow and divide on minimal medium containing BUdR will survive subsequent exposure to UV light. Carlson isolated six auxotrophs that were all leaky, judged by their ability to grow slowly on minimal medium. He suggests that since *N. tabacum* is alloploid, each haploid cell may in fact have been in a diploid state with regard to essential gene copies.

Savage et al. and King et al. (*91, 92*), using haploid cell cultures of *Datura innoxia,* appear to have isolated true auxotrophic mutants, one adenine requiring (Ad1), the second pantothenate requiring (Pn1). These workers used manual selection techniques following mutagenesis with EMS and growth on supplemented medium, to detect these mutant cells lines. These auxotrophs do not appear to be leaky, and as these workers point out, they will serve as starting material for reconstruction experiments in which the efficiency of various selection techniques can be tested.

Thus the future for plant cell mutants appears quite bright. Mutants of potential importance in agriculture have already been obtained: these include maize lines resistant to *Helminthosporium maydis* toxin (*93*), tobacco cell lines resistant to picloram (*72*), and various cell lines with resistance to 2,4-D and to chilling (*68*). The ease with which mutants that overproduce important amino acids and secondary metabolites can be obtained, suggests importance to agriculture and pharmacy. In the latter case, they may well augment or even replace the bacterial strains used at present for such syntheses (*94*).

The Introduction of Foreign DNA into Plant Cells

Genetic Engineering

The term Genetic Engineering is loosely used to describe the process whereby foreign genetic material is introduced into either prokaryotic or eukaryotic cells to form a stable part of the replicating genetic information of these cells. A number of

review articles and books dealing with the topic of genetic engineering have recently been published (*95–97*).

When mammalian cells growing under tissue culture conditions are exposed to foreign DNA, a small subpopulation of these cells stably integrate this exogenous DNA into their chromosomes (*98*). The transforming elements can be maintained within the host genome for hundreds of generations, and frequently express products that alter the phenotypes of the recipient cell. The DNA from viruses or eukaryotic cells has been utilized to transfer genes coding for TK, adenine phosphoribosyl transferase, and HPGRT to mutant cells deficient in these functions (*98*).

Ohyama et al. (*99*) first showed that foreign DNA could be successfully introduced into plant cells. The technique used was to first convert the plant cells into protoplasts, and then to incubate the protoplasts with DNA from *E. coli* or another organism. The usual experimental procedure involved incubating protoplasts with radioactive DNA extracted from *E. coli* for periods of time up to 24 h. After this treatment, the protoplasts were treated with DNAse to remove unbound DNA. Studies showed that up to 3% of the exogenous DNA could be taken up by the cells. Extraction and examination of the donor DNA showed that it had been subjected to considerable degradation by the host cells. Ohyama et al. came to the conclusion that the degraded DNA was not being re-utilized by the host cells.

Other workers in this area have also reported extensive breakdown of donor DNA, but in contrast to Ohyama et al. have obtained evidence that the degraded DNA was re-utilized by the recipient cell. Thus, Uchimiya and Murashige (*100*) carried out a series of experiments using tobacco protoplasts, and DNA from various donors. When the DNA was extracted from the protoplasts after incubation with *E. coli* DNA, it showed greater homology with *E. coli* DNA in hybridization experiments than it did with tobacco DNA. This indicated that the bulk radioactivity associated with the protoplasts could be accounted for by extensive utilization of degraded *E. coli* DNA.

Suzuki and Takebe (*99, 101*) have examined the uptake of single-stranded and double-stranded bacteriophage DNA by tobacco protoplasts. These workers showed that over half of λ-phage DNA taken up was localized in the nucleus, the rest being localized in the chloroplast and cytoplasmic fractions.

Uchimiya and Murashige (*100*) appear to be one of the few groups who have attempted to ascertain if the DNA taken up by recipient cells can be expressed genetically. After incubating tobacco protoplasts of a TMV-susceptible culture, with DNA from a resistant culture that plants regenerated from the protoplasts did not show resistance to TMV.

A second way in which DNA may be introduced into the plant cell genome is via viral infection. The great potential of this technique is shown in work on cultured mammalian cells, such as that carried out by Mulligan and Berg (*102*). These workers have introduced the *E. coli* gene coding for HPGRT into cultured cells of Lesch-Nyhan patients who lack this gene, via transfection with SV40 virus. When the SV40 provides the promotor and the RNA processing signals for the expression of this gene, then the cells are transformed, and can grow in the HAT medium (*102*).

Two vector agents are available at the present time, for the transfection of plant cells, cauliflower mosaic virus (CaMV), and the crown gall bacterium, *Agrobacterium tumifaciens*. CaMV is one of the few plant viruses containing double-stranded DNA, and the DNA when isolated from the virus can infect plants (*103*). Howell et al. (*104*) recently showed that CaMV DNA could be cloned using a bacterial plasmid and then reisolated from the plasmid and used to infect turnip plants (*Brassica napus*). As yet, no foreign genes have been introduced into plant cells using this method.

Crown Gall is a neoplastic disease produced in most dicotyledonous plants by infection with the gram-negative soil bacterium, *A. tumifaciens*. *Crown gall tumors can proliferate autonomously in tissue culture, and in some cases it is possible to regenerate plants from these cells (105)*. It is now firmly established (*106*) that the agent repsonsible for crown gall tumor induction is a large plasmid, the Ti plasmid, contained in all oncogenic strains of *A. tumifaciens*. It is now known (*106*) that the production of the oncogenic state involves transfer of a portion of the Ti plasmid from the bacterium to the plant cell, with the subsequent integration of this DNA into the genome of the transformed cells. The boundaries of the transferred DNA (T-DNA) have recently been cloned from transformed tobacco cells (*106*). It appears that T-DNA is responsible for coding the production of several unusual amino acid derivatives produced by transformed plant cells. These derivatives are collectively termed opines, and include octapine, nopaline, lysopine, histopine, and ornaline (*105*). These derivatives, so produced, appear to be utilized as carbon and nitrogen sources by the bacteria.

The potential use of this system in genetic engineering lies in the possibility of using the plasmid as a vector, and thus being enabled to introduce foreign DNA with specific genetic properties into plant cells. As already indicated, the transformed (tumorized) cells can not only be propagated in cell culture, but when desired, plants can be regenerated from the tumor callus. The cells from these plants still carry the Ti plasmid and synthesize opalines.

Immobilized Plant Cells

The advantages and disadvantages of immobilized bacterial cells as bioreactors have been discussed in a number of recent reviews (*107–109*). By comparison to the literature dealing with immobilized microorganisms, relatively few publications have appeared dealing with plant cells.

Brodelius et al. (*110*) have published work dealing with the immobilization of a number of different species of plant cells on alginate beads. The synthesis of anthroaquinones normally produced by *Morinda citrifolia* in cell suspension culture appeared to be greatly increased when the cells were immobilized. Brodelius et al. (*110*) noted that virtually no increase took place in cell number under the conditions of immobilization. Thus it appeared that nutrients were directed toward production of secondary products at the expense of cell growth and division. The immobilized *M. citrifolia* cells were still apparently viable after 22 days immobilization at 23°C.

The formation of indole alkaloids belonging to the ajmalicine group was studied using immobilized cells of *Catharanthus roseus*. The immobilized cells were fed a mixtrue of ^{14}C-tryptamine and secologanin. After 90 h about 10% of the tryptamine had been converted to a mixture of the three alkaloid isomers, ajmalicine, 19-epiajmalicine, and tetrahydroalstonin. In contrast to cell free extracts that can carry out the same synthesis, the immobilized *C. roseus* cells did not have to be supplied with NADPH.

The biotransformation of the steroid digitoxin to digoxin, was investigated using immobilized *Digitalis lanata* cells. The immobilization did not adversely affect the transformation capacity of these plant cells. This was judged by the increasing concentration of digoxin found in the medium over 33 days.

Alfermann et al. have recently published a study of the biotransformation of cardiac glycosides by *Digitalis lanata* cells immobilized in alginate gel (*111*). These workers showed that immobilized cells of *D. lanata* transform most of the added digitoxin to purpureaglycosde, but deacetyllantoside and digoxin were also produced. β-Methyl digitoxin was readily hydroxylated to β-methyldigoxin by the cells. Both digoxin and β-methyldigoxin were readily excreted into the medium. Although the producing lifetime of the cells was apparently longer when they were immobilized, the biological activity was approximately half that seen under normal batch fermentation conditions. Production of steroids was continued for as long as 61 days, at which time the beads were beginning to disintegrate.

Finally, a review written by Brodelius et al. has been recently published (*112*). This review apparently stresses the potential uses of immobilized plant cells.

Summary

This article has tended to stress some important biotechnological applications of plant cells, as though these lie only in the future. It should be stressed at this point that many Japanese patents already exist describing the utilization of plant cells for many of the types of applications treated in this article. A discussion of these patents, and the subjects to which they apply, can be found in ref (*113*).

Future biotechnological applications of plant cells can conceivably follow in two directions. First, much greater utilization of plant cells using mass growth, and whole cell immobilization techniques already utilized with bacterial cells. Second, the possible creation of new types of cells by the various genetic engineering techniques that have been briefly described in this text. Such techniques may conceivably lead to the production of entirely new and novel compounds by plant cells, or alternatively, may greatly improve the utilization of substrates and the production of existing compounds by these cells.

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