From plant cells to biotechnology*

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The LPC (UNIL) is composed of scientists specialized in the physiology and genetics of plant cells cultivated *in vitro*. They were able to isolate and cultivate cell lines from different plant species producing large amounts of betalains (yellow or violet pigments); The LGB (EPFL) has a large experience in the utilization of bioreactors and the development of biotechnological processes. Both laboratories have now joined their expertise in a co-operative research project seeking to improve the understanding of the physiology and biochemistry of plant cells cultivated in bioreactors and to develop a biotechnological process to produce plant pigments on an industrial scale.

1. Introduction

The first attempts at using plant cell cultures for the production of secondary metabolites date back to the early sixties. Thirty years later the industrial production of enzymes and fine chemicals by plant cells is still limited (1). The most important processes have been established in Japan, one using cell suspensions of *Lithospermum erythrorhizon* for the production of shikonin (a mixture of deep red naphthoquinones used as a colorant for lipsticks and also as an antibacterial agent), the other using cell suspensions of *Coptis japonica* for the production of berberine (used as a tonic and antibacterial agent). In both these processes the cultured cells synthesize the respective products at levels many fold higher than the mother plant. Both compounds command very high market prices, which is essential if the high-tech, high cost fermentation processes used to produce them are to be economically viable. Generally, plant cells cultivated *in vitro* are able to produce secondary metabolites but, in most cases, their accumulation or excretion is too low for an industrial process and mass cultivation has often proven difficult. In this paper we will focus on two aspects of plant cell culture biotechnology in which the progress made during the last few years will, in our opinion, allow a major breakthrough. The first aspect deals with recent developments in the field of plant molecular biology and biochemistry: not only the mastering of DNA technology (gene cloning and gene transfer) but also the discovery of how biosynthetic pathways are regulated at the genetic level. The second aspect is in the field of reactor design (2), where the development of systems for growing large cells (like plant cells) and filamentous microorganisms provide us with new ideas and imaginative concepts.

These new strategies and technologies will be reviewed in the perspective of the project currently developed in our laboratories for the production of betalains, which are naturally occurring yellow (betaxanthins) and violet (betacyanins) pigments found in plants of the order *Caryophyl-*

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* Redakcja dziękuje Autorom i wydawcy Verlag Dr. Felix Wust AG za zgodę na przedruk niniejszego artykułu, który ukazał się w: Swiss Biotech No 1/1991. (Reprint from Swiss Biotech No 1/1991, reproduced by kind permission of Authors and Editor, Verlag Dr. Felix Wust AG).

lales. Structurally, these molecules are composed of a chromophore (betalamic acid) conjugated via an imino linkage to either an amino acid or other amine (the betaxanthins), or to a cyclo–dihydroxyphenylalanine (cyclo–DOPA) residue, which may be glycosylated, (the betacyanins), as shown in Figure 1. Five years ago, the research group at the LPC (UNIL) observed that individual ***Beta vulgaris*** callus cultures contained cells exhibiting a variety of colours, either white (non–pigmented), yellow, orange, red or violet. The range of observed pigmentations was due to the accumulation of betaxanthins, betacyanins or a mixture of both within the cultured cells.

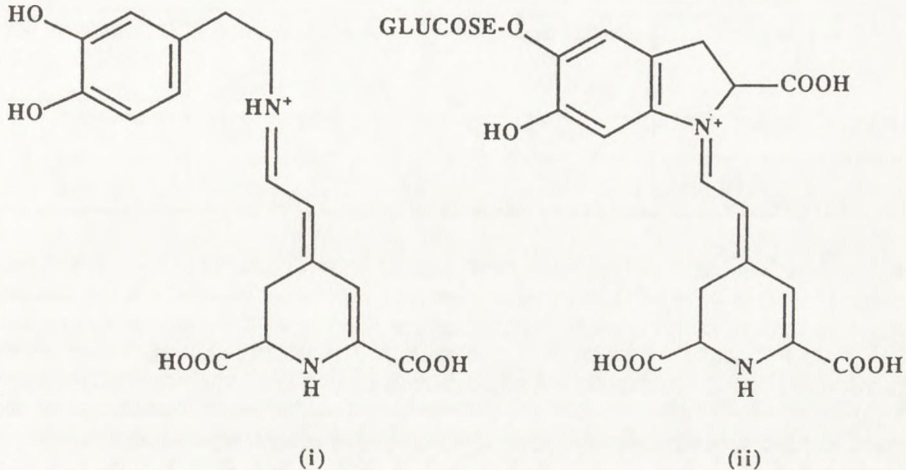

Fig. 1.

At each successive subculture, the differently coloured cell clusters were individually selected and transferred to fresh culture media of different formulation. After several years of repeated selection and media manipulation, stable callus and cell suspension cultures were attained exhibiting uniform cell colour. Genetic analysis of flower pigmentation in the betalain producing plant, *Portulaca grandiflora*, indicates that the number of phenotypes observed in cell culture match exactly the number of genetically predicted phenotypes: cultured cells can express all potential chromo–phenotypes.

2. Regulation of the production of secondary metabolites by plant cells

2.1. Traditional approach

The field of secondary metabolites biochemistry has been, until recently, the "chasse gardée" of natural product chemistry (3) (4). This means that our knowledge of natural products is a large and well founded corpus of chemical structures but that our knowledge of their regulation, is scarce and restricted to only a few examples (e.g. the anthocyanine and flavonoid pathways) (5). Impressive steps have been taken recently with the characterization of enzymes from the indole– and isoquinoline alkaloid pathway (6) and also from the tropane alkaloid pathway (7). The molecular biology and genetics of even the most important biosynthetic pathways has still to be worked out. Our only knowledge in this area is again related to the biosynthesis of anthocyanines and flavonoids (8).

Over the years, we have learned that selection of high yielding cell lines and optimization of culture conditions (media composition, two steps processes...) are both important factors in the establishment of an efficient biosynthetic culture system. These approaches are mainly empirical and do not rely on a strong knowledge of the underlying mechanisms. In some cases, as for the betalain pigments of *Beta vulgaris* (Table 1), it is nevertheless possible to obtain cell suspension cultures which produce specific secondary metabolites in quqntities comparable or even larger than the tissues of the original plant (9), (10).

Table 1

Tissue	Cellular Betaxanthin Content (mg/g dry weight)	Cellular Betacyanin Content (mg/g dry weight)	Total Betalain Content (mg/g dry weight)
Orange cell suspension (BvO)	5,5	0,8	6,3
Violet cell suspension (BvV)	2,9	13,6	16,5
Beetroots (red beet)	0,9	11,8	12,7

The selection process itself has never been studied carefully except in the case of pigment production (11) (12) (13) (14). The fact that an important variability factor exist at the cellular level and that we do not have in most cases analytical procedures of sufficient sensitivity or specificity severely restrict the use of selection as a general tool. The optimization of culture conditions remains the major area in which progress could be made using traditional approaches as well as pursuing the new technologies. In this respect much can be gained by careful use of elicitors (15), addition of biosynthetic pathway precursors (16) and of growth substances.

Two strategies have been proposed and tested in recent years: First, the use of organ cultures (roots, embryos), in place of the classical cell suspension cultures, has been advocated as a means to overcome the organ related specificity of biosynthesis. *Agrobacterium rhizogenes* has been used to induce stable root proliferation; large scale root cultures have been established using specially designed bioreactors (17). In the situation where roots are the natural site of biosynthesis for a desired compound, the method is clearly advantageous. Second, the development of immobilized cultures has allowed biotransformation of selected precursors to be achieved at the industrial level (18).

2.2. New strategies

In the frame of the Lausanne project, investigating the production of pigments by plant cells, some aspects of the regulation of plant cell metabolism will be discussed in the perspective of secondary metabolite production. The establishment of high producing stable plant cell lines is now a reasonable perspective due to the recent progress in the fields of the molecular biology and genetics of plant development.

2.2.1. Constitutive expression of rate limiting enzymes

In primary metabolism (for example the biosynthesis of amino acids) some key enzymes are rate limiting. These enzymes are frequently feed-back regulated by the end-products of the pathway. It is therefore more or less trivial to use mutagenesis, that is selection for resistance to single amino acids or their analogues (19) or site directed mutagenesis, when the DNA-sequence is known, to produce plant cell lines which everproduce a specific metabolite (in this case an amino acid). Whether these methods could be used to stimulate related secondary metabolite synthesis remains to be proved. In the case of *Catharanthus roseus* (20), it has been

shown that tryptophan overproducing cell lines do not produce higher amounts of related alkaloids. This method cannot, unfortunately, be applied to most secondary metabolite pathways: no clear key enzymes have been identified, even when it was observed that the specific activities of each enzyme in a biosynthetic pathway varied extensively. It could nevertheless advantageous, in this situation, to increase the activity of one enzyme as has been suggested for the tropane alkaloid pathway (7). In such a case, the first step is to isolate the specific cDNA by such methods as substractive–hybridization or, more likely, by screening of a cDNA–expression library. This has been done recently for the enzyme hyosciamine–5–β–hydroxylase which is an important enzyme in the production of scopolamine from its precursor hyosciamin (7).

With the same strategy in mind we have, in our laboratory at thr University of Lausanne, isolated the DOPA 4,5–dioxygenase, one of the most important enzymes in the biosynthetic pathway of the betalain pigments, which controls the synthesis of the chromophore betalamic acid from its precursor DOPA (21). We are now attempting the screening of cDNA libraries from higher plants and fungi. Nevertheless it is not always possible to ensure that the increased activity of a specific enzyme will give the desired effect *in vivo*. The second step is to construct an expression vector under the control of a good promoter (CaMV 35S) and to introduce it into a plant cell, either by direct transfer (22) or by *Agrobacterium tumefaciens* mediated transformation. The question over the choice of promoter has yet to be solved: is it best to use a constitutive or an inducible promoter, would a tissue specific promoter be useful?

2.2.2. Introducing new enzymes in plant cells

We should mention here the possibility of itroducing, by transformation, the gene for a missing enzyme in a pathway. The origin of the enzyme could be, in principle, any organism ranging from animals (xenobiotic detoxifying enzymes), microorganisms (yeast) to other plant genera. The group of Heinz Saedler at the Max Planck Institute in Cologne has already succeeded in transferring an enzyme of the anthocyanidin pathway, the dihydroflavonol 4–reductase, from maize to petunia (23). In the case of betalains, the transformation of plant cells with a gene from *Amanita muscaria* for the more stable fungal enzyme DOPA 4,5–dioxygenase could very well increase the rate of synthesis of the chromophore betalamic acid.

2.2.3. Using regulatory genes to increase gene expression

Genes coding for the enzymes of secondary metabolite pathways are expressed as groups in a coordinated and tissue specific way (24). The best documented case being once more the biosynthesis of anthocyanins and flavonoids. It is postulated that such group expression of specific genes is under the control of regulatory genes. To understand what is behind this notion, it is best to look briefly at the genetics and molecular biology of maize. The anthocyanine pathway is an exceptionally good system for studying coordinate gene expression in a plant: all the genes have been cloned, each corresponds to a specific phenotype and the genetics are well known. The regulatory genes: R, B, C, P1 and Vp1 are all necessary for the expression of the Bronze1, C2, A1... locus which encodes the biosynthetic enzymes. The R locus has recently been cloned by the group of Susan Wessler at the University of Georgia (25) and used as a marker for transformation in maize. Under the control of a constitutive promoter, R is able to transactivate the anthocyanin pathway in a homozygous recessive (rr) maize tissue bypassing the tissue specificity. Therefore, in the case of R (and B) alleles, the tissue diversity resides in the ability of the respective promoter to be active in a specific cell type rather than through a differential interaction of the protein products with the regulatory factors. The obvious interest of such regulatory genes is in their ability to switch on a whole group of genes at one time; it is therefore no longer a necessity to genetically engineer each gene coding for a specific enzyme

in a secondary metabolite pathway; only the specific regulatory gene(s) need to be manipulated. It can be expected that loci similar to R, B, C, P1 or Vp1 will be found in other plants; they share together large sequence homology: 80% between R and B. The same R and B loci have cross-kingdom sequence homologies with the *c-myc* human proto-oncogene and therefore identification of similar elements in foreign DNA libraries should be possible.

Such genes placed under the control of constitutive promoters could be transferred to specific cell lines. We can expect therefore to achieve an uncoupling of the biochemical and morphological differentiation in cell suspension cultures. In such stable and overproducing cell lines the isolation and characterization of metabolic intermediates and enzymes should be easier. The expected broad specificity of regulatory genes will probably allow transpecific or even transgeneric transformations to be efficient. If this proves to be true, this will mean that uncommon or neglected plants could be made to produce, with a minimal amount of experimentation, large amounts of new and valuable products.

3. Development of biotechnological processes

Critical to the development of a process employing cell culture systems is an exact understanding of the relationship between culture growth and product formation. In the majority of cases, the phase of active cell growth (exponential phase) is associated with little or no product synthesis, while high rates of metabolite production are associated with low rates of cell growth (stationary phase). In some cell systems however, growth and product synthesis are not mutually exclusive, and the production of the desired metabolite occurs in the exponential phase of growth (26). Furthermore, genetic engineering techniques may be used to modify the natural coupling between growth and product synthesis, thereby facilitating biotechnological exploitation.

Selecting the most appropriate bioreactor and cultivation technique for optimal culture growth and metabolite production is one of the most important problems to be solved. Many data are at present available on the growth and production kinetics of many plant cell cultures. However, most are not suitable for the design of large-scale processes because of the experimental set-up used to collect data: mostly batch cultures in shake flasks. Under such conditions, cells are exposed to a continually changing physical and chemical environment resulting in continuous variation of culture growth rate throughout the cultivation cycle. The gas phase also changes continuously: O_2 concentration can decrease from 21% to less than 13% and CO_2 may rise up to 11%. Therefore, growth and production kinetics must be studied under well defined conditions and at different steady states, using the type of bioreactor planned for the large scale process.

3.1. Batch cultivation and immobilized cells

If growth and metabolite production are not directly associated, then batch, fed batch or two-stage cultivation techniques are the most suitable. Fed batch processes allow higher biomass concentration to be attained, resulting in increased metabolite production. If plant cells require different conditions and/or media for proliferation and secondary product synthesis, then a two-stage process could be advantageous. Cell immobilization techniques may be used to extend the period of metabolite production, but are generally only useful where the desired metabolite is released from the cell into the culture medium.

Immobilized cell systems are claimed to present several advantages over suspension culture methods: reduction of shear stress, reconstitution of a pseudo-tissue or organ mimicing the environment of a differentiated cell *in vivo*, prevention of cell aggregation at the liquid-air interface, and easier separation of the biomass from the broth.

To date, most work with gel entrapped plant cells has been undertaken in shake flasks sys-

tems of culture volume 50 to 500 ml, while studies on scale–up into large volume bioreactors remain in their infancy. Different techniques have been developed to immobilize plant cells at small scale, but their use in industrial processes could be difficult because of several drawbacks, which include crosslinking agent toxicity (glutaraldehyde), chelation by medium ions, limited mechanical stability and inherent difficulties in large–scale operation. For example, calcium alginate entrapment appears to be a simple, cheap, reproducible and mild technique. but its industrial application is somewhat questionable because of difficulty in producing large amounts of standardized alginate beads. Furthermore, mechanical stability of the gel is poor due to the chelation of calcium by phosphate, and may also be affected by the growth of the cells. Kappa–carrageenan is less sensitive to chelating agents, but the production of large amounts of uniform spherical particles is difficult and probably too expensive. If agar or agarose is used, the need for relatively high temperatures could be deleterious to plant cells and the mechanical strength of the gel is insufficient for industrial purposes.

Immobilization onpolyurethane foam is a single step process, which means less risk of microbial contamination, but the requirement for long culture periods prior to the actual production of metabolites is not acceptable from a commercial point of view. The use of porous beads could be problematic at the level of controlling microenvironmental conditions, unless pore size and particle diameter can be strictly controlled. In addition, the adhesion of plant cells to the surface of such carrier particles is not very strong, limiting the application of this technique. Micro–encapsulation of cells in different inert materials appears to be a promising method, permitting control over aggregate size, improving cell–to–cell contact and allows high cell densities to be attained. At the present time however, there are no reports on the production of secondary metabolites from cultured plant cells using this technique.

Interesting results have been obtained with plant cells immobilized in small scale membrane reactors (hollow–fibres, spiral wound units, flat plate, tubular or multimembrane reactors). Plant cells are physically separated from the liquid production medium by a membrane, which allows substrates, nutrients and cell products to pass freely between cells and culture medium. Some of these systems are easily scaled–up, but problems could arise during their long term operation because of the heterogeneous nature of the culture and the progressive fouling of the membranes. This could lead to reduced mass transfer and diffusional limitations on gases, nutrients and products to and from the plant cells, thus affecting the activity and the viability of the partialy differentiated cells in the center of the aggregate. Furthermore, the manual loading of such systems is difficult and the immobilization procedure presents an extra risk of contamination.

The use of fibrous polyester sheets as an immobilization support for cultured plant cells was reported recently. Early data from experiments with this material, which has the advantage of a high surface to volume ratio, suggest this to be a promising technique (27). Such surface immobilization procedures do not impose a physical barrier to mass transfer other than diffusional limitations occurring within the biofilm and it seems to approximate, more than other culture modes, the true tissue culture of plant cells. This system has been successfully tested at small scale in a modified airlift and a classical stirred vessel using polyester sheets inserted in a vertical spiral configuration.

One of the obvious difficulties in working with immobilized plant cells is stimulating the release of intracellularly accumulated products without loss of cell viability. This is an absolute requirement if biomass is to be used over extended culture periods during (semi–)continuous process operation and for product recovery. Different techniques have been described to stimulate such release: pH variations, temporary and reversible permeabilization of the cells by the addition of a chemical (dimethylsulfoxide or solvent), or exposure to ultrasound.

Further improvements are required however, before the use of immobilized plant cells for the complete biosynthesis of secondary metabolites at an industrial scale can be realized. The commercial exploitation of immobilized techniques will require extra investment on the part of in-

dustry, but since the improvement in productivity over suspension culture methods is often only marginal, the interest in such techniques could be seriously limited from an economic point of view. At present it appears that the major role of this technique will be in the biotransformation of precursors into more commercially interesting products.

3.2. Continuous culture and suspended cells

If production of the desired metabolite is growth associated then continuous cultivation of freely suspended cells, under steady-state conditions, in a reactor that imparts low hydrodynamic stress is probably the most suitable. A pulsed substrate mode of culture could also be appropriate to maximize product formation by intermittent addition of certain nutrients or growth regulators. The use of the recently developed perfusion technique (spin-filter or wire cage) could also be interesting under certain conditions and with the appropriate adaptations. Moreover, the retention of actively growing cells in the reactor without limiting the hydraulic dilution rate could offer a promising alternative to that of the classical technique.

Continuous culture using the same type of bioreactor as planned for the large scale process is a convenient methodology, since growth and production can be studied undre several steady state conditions: glucose or nitrogen limited cultures, different dilution rates, etc. (28). It is first necessary to determine the most efficient protocol for achieving high cell densities and the type of bioreactor best suited for mass cultivation of the cell line under investigation. Indeed, O_2 requirement, shear sensitivity, rheological characteristics and the size of the aggregates vary from one culture to another. It is therefore likely that a single system is not applicable to all plant cells because of the wide variety of culture types and growth requirements. A good reactor will provide efficient oxygenation under conditions of low hydrodynamic stress, control over environmental conditions (nutrient concentration and aggregate size), safe aseptic operation for a long period of time, easy scale-up and operational simplicity.

For a long time, plant cell suspensions have been regarded as sensitive to shear due to their large size, their rigid cell wall and their tendency to form aggregates. Therefore, low shear reactors with adequate agitation and aeration were used, such as airlift reactors or bubble columns. Continuous stirred tank reactors (CSTR) were almost precluded since several regions of high shear are produced by the impeller rotation, particularly in the flow stream, the trailing vortices behind the impeller blades and the turbulent breakup of the laminar flow from the impeller. It has been shown recently however, that cell lines of *C. roseus* are tolerant to a stirrer speed of 1000 rpm and able to grow at 150-200 rpm (29). The cell aggregate size decreases, but the cell viability is not affected. It has been shown for *Helianthus annuus* cell suspensions cultured in different vessels that the growth rate, doubling time and final biomass were similar for shake flasks, airlift and stirred reactors (30).

This is extremely important, since the ability to use a standard CSTR for the cultivation of many different plant cells would allow the use of existing bioreactors, with only a few modifications, rather than the construction of specific reactors: the impeller stirred bioreactor is the most commonly used in the fermentation industry, facilitating the introduction of a plant cell process. In addition, CSTRs allow the separate control of culture agitation (stirred speed) and aeration, and are associated with reduced foaming and meringue formation.

The content of most industrial-scale bioreactors is agitated by the rotation of flat-bladed impellers, also called Rushton turbines, which are generally about 1/3 of the reactor diameter. The region close to the impeller is characterized by an intense high speed vortex behind each blade, which is a region of low pressure and high shear stress. A decrease in agitation speed is therefore required in large scale reactors to reduce the velocity of the impellers tips and the shear stress. With such turbines there is also poor exchange flows between one agitator and an another, which could result in top to bottom variations in pH, dissolved O_2 or nutrient concentra-

tion. Thus the growing cells may encounter a continually changing environment as they move around large bioreactors. Impellers with increased width or diameter (large flat–bladed) and/or improved design (paddle–type systems, hydrofoil agitators or marine impellers) produce lower shear at their tips, give better top to bottom mixing, and allow better homogeneity, heat transfer and air–handling capacity (31) (32).

The size and design of impellers and the effect of baffle plates need to be defined and optimized for each cell line in order to determine the relationship between shear, culture growth, aggregate size and metabolite production. It is noteworthy that cells in the center of large aggregates may be altered in their biochemical functions by O_2 and/or nutrient limitations. For example, thiophene production by cell suspensions of *Tagetes patula* increases with increasing aggregate diameter with an optimum at 11–13 mm (33).

The recent development of a bubble–free aeration system in a stirred reactor provides a suitable technique to improve gas transfer without inducing cell damage through shear stres (34). In this system of aeration, air flows through porous membrane fibers connected to silicone tubes at wall entrances in the cover plate of the reactor. By increasing the proportion of O_2 in the inlet gas mixture, a pO_2 of up to 30% can be maintained without generating air–bubbles, and with only gentle mixing of the cell suspension.

New agitation systems, comparable in their principle to airlift, but with liquid (and not air) circulation, have been successfully tested with different cell lines and appear to be promising alternatives for the cultivation of plant cells (35) (36). In one case, the rotation of a cell–lift impeller drives an internal circulation and creates a negative pressure at its tips, drawing an internal circulation and creates a negative pressure at its tips, drawing the cells through a draught tube where they exit and flow down the side of the vessel. Exit ports at the top of the draught tube can be used in either a radial or a tangential configuration. Air is supplied through a fritted glass filter or a ceramic air stone. Such a cell–lift impeller system results in a smaller average aggregate, and therefore avoids the problems of reactor port fouling and poor mixing caused by large cell aggregates. Above 100 rpm however, the cell–lift impeller produces surface turbulence resulting in the formation of a meringue of deposited cells on the glass. Another system, the liquid–impelled loop reactor, is based on the introduction and the circulation of an exogenous organic solvent, not miscible with water. The movement of the solvent in the reactor and density difference will cause circulation and mixing of the suspension.

Secondary metabolites may be recovered from continuous plant cell cultures using destructive methods, like cell harvesting and disruption, followed by the purification of the product of interest. Non destructive methods are preferable however, thus allowing further product synthesis and repeated cycles of production and harvest from each culture. Cavitational events caused by continuous–wave ultrasound induces pigment efflux *B. vulgaris* cells without affecting their viability, thus allowing repeated cycles of production and release, at least on a small scale (37). Optimization of the frequency, power, duration of sonication and harvest frequency would be necessary for each different cell line used.

4. Plant cell biotechnology in Lausanne

The present cooperative research programme of the LPC (UNIL) and the LGB (EPFL), is directed toward determination of the kinetics of growth and pigment production in cell suspensions of *B. vulgaris* selected for their high productivity of betalains (Figure 2). The project involves the mass cultivation of these high producing cell suspensions in bioreactors, with the aim of optimizing growth conditions during scale–up of culture volume and in bioreactors of different operating principles. The bioreactors to be studied include both air–lift and stirred tank systems. This work will also examine the effects of bubble free aeration in an attempt to eliminate the problems of hydrodynamic shear stress and meringue formation (wall growth) which are often

encountered when culturing plant cells, particularly at high cell densities. Parameters to be studied include aeration/sparging systems, impeller design, mass oxygen transfer rates (Kl_a) and cellular oxygen demand. The LPC will continue in parallel to work on the biochemistry and molecular genetics of this model system to improve the basic understanding of the regulation of secondary metabolite biosynthesis.

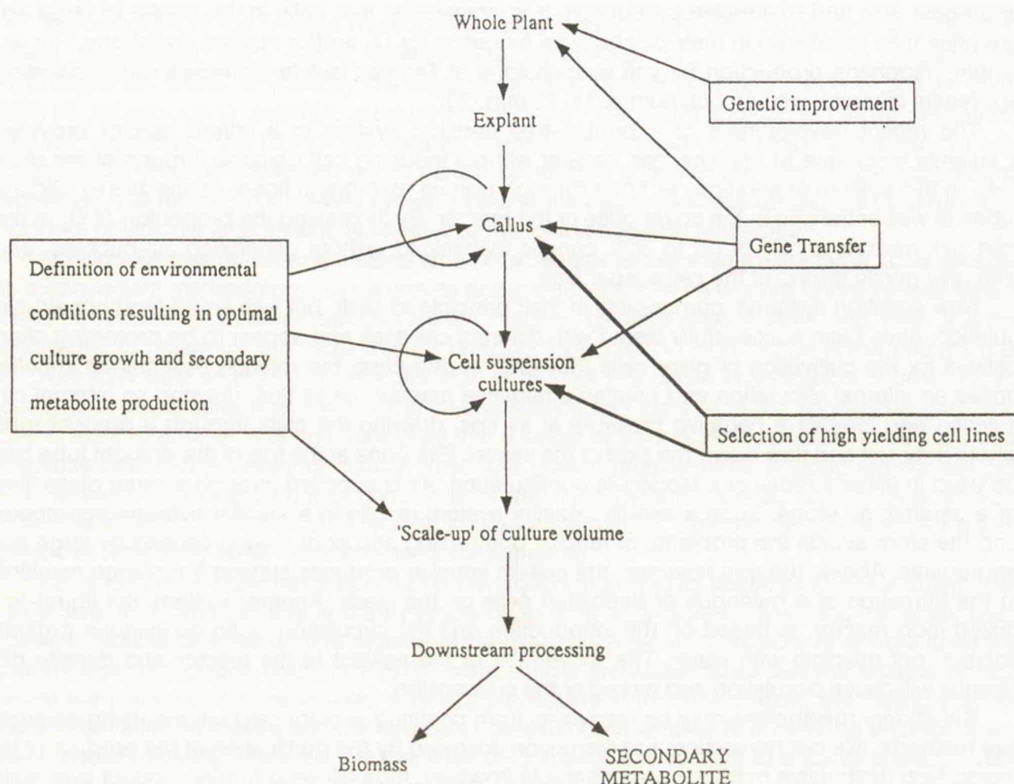

Fig.2

This common work, initially supported by the UNIL-EPFL Research Foundation, will be improved and reinforced for the next three years with the joint support of the Swiss Commission for the promotion of scientific research and an industrial partner. This study of feasibility and development of biotechnological processes will be undertaken using the approaches described above, with the ultimate goal of the development of an industrial scale process which uses *in vitro* cultured plant cell suspensions in the commercial production of betalains for the food industry. It has been proposed that, in the light of recently highlighted health and safety problems with certain synthetic food colorants like tartrazine, and the general public feeling toward artificial food additives, that the betalains may be suitable as natural replacements for some water soluble food colorants in current use.

In this survey we bring to focus some of the prospects for the large scale production of secondary metabolites by plant cell cultures. It seems necessary to increase our manpower and

financial investment in the study of the genetics and molecular biology of the regulation of plant development in relation to the biosynthesis of secondary metabolites. Tools are available which could be used to overcome some of the existing difficulties encountered with plant cell cultures. The large number of valuable known and still unknown products synthesized by plants is worth our efforts in the long term. The new prospects offered by the proposed genetic manipulations and biotechnological processes could very well restore interest in the use of plant cells for the production of metabolites when balanced against the use of microorganisms. This perspective should therefore encourage people involved in basic research to cooperate with those actively developing technical and commercial applications.

References

1. Zrÿd JP. Cultures *in vitro* et production de métabolites secondaires. In: Zrÿd JP ed. Culture de cellules, tissus et organes végétaux. Lausanne: Presses Polytechnigues Romandes, 1988: 228–234.
2. Kreis W, Reinhard E. The production of secondary metabolites by plant cells cultivated in bioreactors. Planta Med. 1989; 55: 409–416.
3. Mann J. Secondary metabolism (2 ed.). Oxford UK: Oxford Science, 1987: 374.
4. Herbert R. The biosynthesis of secondary metabolites (2 ed.), London: Chapman and Hall, 1989: 229.
5. Luckner M. Secondary metabolism in microorganisms, plants, and animals. Berlin: Springer, 1989.
6. Zenk MH, Rueffer M, Amann M, Deus-Neumann B, Nagakura N. Benzylisoquinoline biosynthesis by cultivated plant cells and isolated enzymes. J. Nat. Prod. vol. 48 P725 1985; 48: 725–738.
7. Yamada Y, Hashimoto T. Possibilities for improving yields of secondary metabolites in plant cell cultures. In: Nijkamp HJJ, van Aartrijk J ed. Progress in Plant Cellular and Molecular Biology. Amsterdam: Kluwer Acad., 1990: 547–556.
8. Halbrock K, Grisebach H. Enzymic controls in the biosynthesis of lignin and flavonoids. Annu. Rev. Plant Physiol. 1979; 30: 105–130.
9. Böhm H, Rink E. Betalains. In: Constabel F, Vasil I ed. Phytochemicals in plant cell cultures. San Diego: Academic Press, 1988: 449–466.
10. Girod PA, Zrÿd JP. Secondary metabolism in cultured red beet (*Beta vulgaris* L.) cells: differential regulation of betaxanthin and betacyanin biosynthesis. Plant Cell, Tissue and Organ Culture 1990; in press.
11. Girod PA, Zrÿd JP. Clonal variability and ligft induction of betalains synthesis in red beet cell cultures. Plant Cell Rep. 1987; 6: 27–30.
12. Tabata M, Ogino T, Yoshioka K, Yoshikawa N, Hiraoka N. Selection of cell lines with higher yield of secondary products. In: Thorpe TA ed. Frontier of Plant Tissue Cultures. Calgary: UNiv. Calgary Press, 1978: 213–222.
13. Tabata M, Fujita Y. Production of shikonin by plant cell cultures. In: Zaitlin M, Day P, Holleander A ed. Biotechnology in Plant Sciences. Orlando Florida: Acad. Press, 1985: 207–218.
14. Dougall DK, Johnson JM, Whitten GH. A clonal analysis of anthocyanin accumulation be cell cultures of wild carrots. Planta 1980; 149: 292–297.
15. Eilert U, Kurtz W, Constabel F. Stimulation of sanguinarine accumulation in *Papaver somniferum* cell cultures by fungal elicitors. J. Plant Physiol. 1985; 19: 65–66.
16. Tam W, Kurz W, Constabel F, Chatson KB. Biotransformation of thebaine by cell suspension cultures of *Papaver somniferum* cv. Marianne. Phytochemistry 1982; 21: 253–255.
17. Kamada H, Okamura N, Satake M, Harada H, Shimomura K. Alkaloid production by hairy–root cultures in *Atropa belladonna*. Plant Cell Rep. 1986; 5: 239–242.
18. Alfermann AW, Schuller I, Reinhard E. Biotransformation of cardiac glycosides by immobilized cells of *Digitalis lanata*. Planta Med. 1980; 40: 218–223.
19. Widholm Jm. Selection of mutants which accumulate desirable secondary compounds. In: Constabel F, Vasil IK ed. Cell Cultures in Phytochemistry. San Diego: Academic Press, 1987: 125–137.
20. Schallenberg J, Berlin J. 5–methyltryptophan resistant cells of *Catharanthus roseus*. Z. Naturforsch., C: Biosc. 1979; 34C: 541–545.
21. Girod PA, Zrÿd JP. Biogenesis of betalains: purification and partial characterisation of DOPA 4,5–dioxygenase from *Amanita muscaria*. Phytochemistry 1990; in press.

22. Paszkowsky J, Shillito RD, Saul M, et al. Direct gene transfer to plants. EMBO J. 1984; 3: 2717-2722.
23. Meyer P, Heidmann I, Forkmann G, Saedler H. A new Petunia flower color generated by transformation of a mutant with a maize gene. Nature 1987; 330: 677-678.
24. Zrÿd JP. Quelques aspects de la biologie des métabolites secondaires des plantes. Revue Horticole Suisse 1990; 62(1): 24-27.
25. Ludwig SR, Bowen B, Beach L, Wessler SR. A regulatory gene as a novel visible marker for maize transformation. Science 1990; 247: 449-450.
26. Scragg AH, Cresswell RC, Ashton S, York A, Bond PA, Fowler MW. Growth and alkaloid production in bioreactors by a selected *Catharanthus roseus* cell line. Enzyme Microb. Technol. 1989; 11: 329-333.
27. Archambault J, Volesky B, Kurz WGW. Production of indole alkaloids by surface immobilized *C. roseus* cells. Biotechnol. Bioengin. 1990; 35, 660-667.
28. van Gulik WM, Meijer JJ, ten Hoopen HJG, Luyben KCAM, Libbenga KR. Growth of a *Catharanthus roseus* cell suspension culture in a modified chemostat under glucose-limiting conditions. Appl. Microbiol. Biotechnol. 1989; 30: 270-275.
29. Scragg AH, Allan EJ, Leckie F. Effect of shear on the viability of plant cell suspensions. Enzyme Microb. Technol. 1988; 10, 361-367.
30. Scragg AH. Large scale cultivation of *Helianthus annuus* cell suspensions. Enzyme Microb. Technol. 1990; 12: 82-85.
31. Hooker BS, Lee JM, An G. Cultivation of plant cells in a stirred vessel: effect of impeller design. Biotechnol. Bioengin. 1990; 35: 296-304.
32. Nienow AW. Agitators for mycelial fermentations. TIBTECH 1990; 8: 224-233.
33. Hulst AC, Meyer MMT, Breteler H, Tramper J. Effect of aggregate size in cell cultures of *Tagetes patula* on thiophene production and cell growth. Appl. Microbiol. Biotechnol. 1989; 30: 18-25.
34. Piehl G-W, Berlin J, Mollenschott C, Lehmann J. Growth and alkaloid production of a cell suspension culture of *Thalictrum rugosum* in shake flasks and membrane-stirrer reactors with bubble free aeration. Appl. Microbiol. Biotechnol. 1988; 29: 456-461.
35. Treat WJ, Engler CR, Soltes EJ. Culture of photomixotrophic soybean and pine in a modified fermentor using a novel impeller. Biotechnol. Bioengin. 1989; 34: 1191-1202.
36. Buitelaar RM, Susaeta I, Tramper J. Application of the liquid-impelled loop reactor for the production of anthraquinones by plant cell cultures. In: Nijkamp HJJ, van der Plaas LHV, van Aartrijk J ed. Progress in Plant Cellular and Molecular Biology. Amsterdam: Kluwer Acad., 1990: 694-699.
37. Kilby NJ, Hunter CS. Repeated harvest of vacuole-located secondary product from in vitro grown plant cells using 1,02 MHz ultrasound. Appl. Microbiol. Biotechnol. 1990; 33, 448-451.

Od komórki roślinnej do biotechnologii

Streszczenie

Biotechnologia komórek roślinnych rozwija się w oparciu o postęp wiedzy w zakresie biologii komórki, genetyki, biologii molekularnej i fitochemii oraz wykorzystuje nowe techniki laboratoryjne i osiągnięcia inżynierii hodowli komórek *in vitro*. Nowe strategie tego rodzaju w zakresie doskonalenia linii komórkowych i opracowywania procesów technologicznych są przedmiotem niniejszego opracowania. Regulacja ekspresji genów, konstytutywna ekspresja genów „wąskich gardeł" szlaków biosyntezy i wprowadzanie genów kodujących nowe dla komórki enzymy - to najbardziej obiecujące dla biotechnologii kierunki genetyki komórek roślinnych. Artykuł zawiera także analizę warunków hodowli okresowej i ciągłej komórek roślinnych w zawiesinie oraz procesów z użyciem komórek immobilizowanych. Pracę kończy omówienie badań własnych, prowadzonych nad biotechnologią wytwarzania barwników w hodowli komórek *Beta vulgaris*.

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