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in

Demarly Y. (ed.).
Place et rôle des biotechnologies dans les systèmes de recherche agronomique des pays méditerranéens

Zaragoza : CIHEAM
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 14

1991
pages 59-74

Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects

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SUMMARY - Plant secondary metabolites are economically important, in such different fields as drugs, fragrances, pigments, food additives and pesticides. An alternative to their extraction from the plant, is to synthesize them from in vitro cell cultures. After a brief revision of the historical background of the in vitro production, its technical aspects according to its diverse forms, along with a number of applications including industrial-scale products, illustrate this process. Economic data, with the indication of limiting factors and an analysis of potential valuable developments, complement the assessment of this new process. The market share, the investment-profit ratio, the competition with other applications will also be valuable data for decision-making.

Key words: Secondary metabolites - Tissue culture - Elicitor cell suspension culture - Bioconversion - Biomass - Immobilized cells - Pigments - Fragrances - Pharmaceutical compounds.

RESUME - "Production de substances biochimiques d'intérêt par culture de cellules de plantes supérieures : aspects biotechnologiques et économiques". Les métabolites secondaires chez les végétaux présentent un grand intérêt économique, dans des domaines aussi divers que la pharmacie, les arômes, les pigments, les additifs alimentaires et les pesticides. Une alternative à leur extraction à partir de la plante, consiste à les faire produire par des cultures cellulaires in vitro. Après un bref rappel historique de cette production in vitro, les aspects techniques de ses diverses formes ainsi que de nombreux exemples allant jusqu'à des productions industrielles dans les divers domaines illustrent le procédé. Des données économiques accompagnées d'une mise en évidence des facteurs limitants et d'une analyse des développements positifs possibles complètent l'évaluation de cette voie nouvelle de production. Le volume du marché, les rapports investissements-bénéfice, la compétitivité avec d'autres voies devront également être des éléments importants de décision.

Mots-clés : *Métabolites secondaires - Culture de tissus - Suspensions cellulaires éliciteurs - Bioconversion - Biomasse -Cellules immobilisées - Pigments - Arômes - Substances pharmaceutiques.*

Plant secondary metabolites

The distribution of secondary metabolites in plants is far more restricted than that of primary metabolites; a compound is often only found in a few species, or even within a few varieties within a species. Though their function in plant metabolism is unclear, nevertheless they may have an ecological role, e.g. as sexual attractants for pollinating insects or in defence mechanisms against predators (Grisebach, 1988). Secondary metabolites often accumulate in the plant in small quantities, sometimes in specialized cells. Hence their extraction is often difficult. Among them are many compounds which are commercially important as medicinal substances, fragrances, food additives (pigments, flavouring and aromatic compounds) and pesticides (Heble and Chadha, 1985b; Kurz, 1989).

In spite of the progress made in organic synthesis or semi-synthesis of a wide range of compounds similar to those produced by the plants, extraction of secondary metabolites from plants is still of considerable commercial importance. A large number of these metabolites are difficult or virtually impossible to synthesize at economic values. In several cases, the natural product is more easily accepted by consumers than an artificially produced one. Both reasons, objective and subjective, explain that natural extraction still applies to a large number of aromas or fragrances which are the result of a mixture of hundreds of different compounds as is the case of jasmine and strawberry, or to biochemicals that have complex molecular structures (e.g. some alkaloids and glycosides).

There is great interest in developing alternatives to the intact plant for the production of plant secondary metabolites. This originally had centred on the use of tissue and cell cultures though the most recent approaches involve applying molecular biology techniques to enhance the metabolic pathways leading to specific compounds. During the past three decades, research has concentrated on the use of plant cell and tissue cultures, particularly in Japan and Germany, but also to a lesser extent in the USA, for the commercial production of a wide range of secondary metabolites, in just the same way as bacteria and fungi have been used for antibiotic or amino-acid production (Sasson, 1988; Kurz, 1989).

Plant cell cultures: advantages and drawbacks

Plant tissue cultures were first established in 1939-1940. However, it was only in 1956 that the first patent for the production of metabolites by mass cell cultures was filed by the American pharmaceutical company, Pfizer Inc. (Pétiard and Bariaud-Fontanel, 1987). The potential of plant cell cultures to produce useful compounds, especially for drug development, was perceived in the late 1960s. Thus Kaul and Staba (1967) and Heble et al. (1968) isolated visnagin and diosgenin respectively from cell cultures in larger quantities than from the whole plant. However, a large number of cultures failed to synthesize products characteristic of the parent plant. For instance, morphinan, tropane and quinoline alkaloids are synthesized only at extremely low levels in cell cultures (Berlin, 1986). That is why, after a surge of interest, the trend of research declined.

In 1976, at an international congress held in Munich, Zenk and his co-workers demonstrated the outstanding metabolic capacities of plant cells and highlighted the spontaneous variability of plant cell biosynthetic capacity, which could explain the contradictory results obtained earlier. This natural variability is exploited to identify high-yielding cultures for use on an industrial scale (Tabata *et al.,* 1976; Zenk *et al.,* 1977; Zieg *et al.,* 1983; Yamada, 1984; Benjamin *et al.,* 1986). Since the late 1970s, research and development in this area has seen a high increase in the number of patent applications filed, especially by the scientific and corporate sectors in the Federal Republic of Germany and Japan. In 1983, for the first time, a dye, shikonin, with anti-inflammatory and anti-bacterial properties, was produced by plant cell cultures on an industrial scale by Mitsui Petrochemical Industries Ltd (Fujita *et al.,* 1982). However, although this was thought to be a major breakthrough, shikonin production is still (in 1990) the only plant product to be produced on a commercial scale by cell cultures.

Confronted with having to increase the amount of secondary metabolites in plant cell cultures, the need for greater biochemical and molecular research on the secondary metabolism of plants has been frequently emphasized (Fowler, 1981, 1984a,b; Misawa, 1985; Berlin, 1986; Stafford *et al.,* 1986). The results of research in this area could lead to the successful manipulation of secondary metabolism and significantly increase the amounts of the compound(s) sought.

It is now thought that any substance of plant origin can be produced by cell cultures. Thus it should be possible to achieve the synthesis of a wide range of compounds such as alkaloids, flavonoids, terpenes, steroids, glycosides, etc., i.e. a total of several hundreds with complex chemical structures, using plant cell culture technology. It also seems possible to identify cell lines that can produce amounts of compounds equal or even higher than those in the plant from which they derive. Furthermore, new molecules which have not been found previously in plants or have even been synthesized chemically, have been produced by cell cultures. Thus this technology constitutes a genuinely new means of achieving production of novel metabolites.

Finally, plant cells can transform natural or artificial compounds, introduced into the cultures, through a variety of reactions such as hydrogenation, dehydrogenation, isomerization, glycosylation, hydroxylation, opening of a ring and addition of carbon atoms (Rajnchapel-Messai, 1988). On the other hand, growing plant cells on a large scale would permit a stricter control of the quality of the products as well as their regular production without dependence on the variations of natural production resulting from climate and socio-political changes in their countries of origin.

The techniques of plant cell cultures include the following sequential stages or developments: selection among wild plants of a high-producing one, *in-vitro* culture or callogenesis, which involves the selection and stabilization of producing calli with a view to identifying a high-producing line or strain; maximizing callus or cell suspension, culture conditions and isolation of the best-producing line; industrial scaling-up, mass cultivation in bioreactors; downstream processing, i.e. extraction and purification of the compounds sought.

Means for increasing plant secondary metabolites

There are several means of increasing the production of secondary metabolites by plant cell cultures or suspensions. These are:

- use of biotic or abiotic elicitors that could stimulate the metabolic pathways as in the intact plant;
- addition of a precursor of the desired compound in the culture medium with a view to increasing

its production or inducing changes in the flux of carbon to favour the expression of pathways leading to the compound(s) sought, i.e. alteration of controls of secondary metabolism pathways;

- production of new genotypes through protoplast fusion or genetic engineering but this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression once introduced in the plant cells;
- use of mutagens to increase the variability already existing in living cells;
- use of root cultures.

The use of mutagens could increase the naturally-occurring diversity among the cell clones and induce the creation of new higher-producing lines or strains. Also cell fusion and gene transfer could improve the synthesis capacity of cells. However, the results of these efforts depend on the knowledge of the metabolic pathways and their genetic control.

1. Alteration of controls of secondary metabolism pathways

For the majority of secondary metabolism pathways, the proposed biosynthesis, deduced from chemical considerations and feeding experiments, must await verification by the identification of the corresponding enzyme reactions. Thus, direct manipulations of the pathways are not possible due to the lack of enzymological background (Berlin, 1988). Enzymological knowledge relating to secondary metabolism pathways has been drawn from the studies of cell suspension systems (Hahlbrock and Grisebach, 1979; Zenk, 1980; Zenk *et al.,* 1985) but organ cultures and intact plants are excellent sources for such enzymes.

Another issue related to the possible alteration of secondary metabolism pathways concerns the identification of those enzymes which are regulatory or rate-limiting. This is most probably the case for enzymes at the beginning of a sequence or at branching points of metabolic pathways, especially when their absence prevents *de novo* formation of the compounds or when manifold increases in the activity of these enzymes are observed under conditions promoting a major increase in product formation. It is also necessary to know whether the other biosynthetic enzymes are co-induced with the proposed regulatory enzyme or are permanently present even in non-producing cell cultures. Such knowledge helps to evaluate the possibility of specifically manipulating these secondary metabolism pathway(s) to increase the concentration of the desired product.

It is generally assumed that the regulatory genes occur as gene families and that each gene within one family is separately controlled and is thus expressed by a different signal. Thus, direct alterations of such controls would be difficult to achieve (Berlin, 1988). It is, however, possible to introduce a desired gene into an intact plant through suitable vectors and have the gene expressed in a specific organ such as the chloroplasts (Broeck *et al.,* 1985; Fraley *et al.,* 1985; Klee *et al.,* 1985; Potrykus *et al.,* 1985; Abel *et al.,* 1986; Eckes *et al.,* 1986; Schocher *et al.,* 1986; Shah *et al.,* 1986). The gene coding for chalcone synthase (CHS) could be the first plant gene to be used in such a transformation process to study its interference with a secondary metabolism pathway. Veltkamp and Mol (1986) have carried out experiments aimed at repairing anthocyanin biosynthesis in mutants deficient in CHS through genetic transformation.

Although there is no example of altering the regulatory controls of secondary metabolism pathways through genetic engineering (with a view to improving the production rates in cell cultures of a desired compound), this seems to be the only promising approach. To achieve this end, it is crucial that more gene coding for biosynthetic enzymes with a regulatory function in secondary metabolism pathways should be identified. Another approach which might be more relevant biotechnologically would be to transfer plant genes into bacteria and to express them for stereospecifically difficult biotransformations (instead of organic synthesis). Once the genes for the relevant enzymes are isolated and cloned, it could then be decided whether transformed plant cells, transformed micro-organisms or immobilized enzymes are the best for increasing the production of a specific compound (Berlin, 1988).

2. Effects of elicitors

The use of abiotic and biotic elicitors is a promising tool to improve the yields of products in cell-culture systems (DiCosmo and Misawa, 1985). Biosynthesis of flavonoids is induced by light via phytochrome or/and UV-photoreceptors and by infection with phytopathogenic organisms or compounds which induce the synthesis of antimicrobial compounds in plants. The addition of an extract of *Verticillium,* a parasitic fungus of plants, has induced the synthesis of gossypol by cell suspensions of *Gossypium arboreum* (Heinstein, 1984, in Pétiard and Bariaud-Fontanel, 1987). See also Cramer *et al.* (1985).

It can be predicted that very specific elicitors should be found which will significantly improve the amounts of morphinan, tropane, or quinoline alkaloids in plant cell cultures (Berlin, 1988). The increase in the production of morphinic alkaloids by poppy cells could be likewise increased but this is still an empirical

procedure and a single general rule cannot be formulated. The elicitor needs to be added regularly and this therefore complicates its use.

3. Bioconversion

Plant cells can transform a wide range of substrates and thus perform several reactions such as oxidation, hydroxylation, reduction, methylation, glucosylation, acylation and amino-acylation (Furuya, 1978; Heble *et al.*, 1983; Heble and Chadha, 1985b).

One could cite the transformation of steviol by cells of *Stevia rebaudiana* into a glycoside, steviobioside, which is 300 times sweeter than sucrose and which is used as a sweetener in Japan. Another example is that of salicylic acid which is glycosylated by cell cultures of *Mallotus japonica* to give a product having a higher analgesic power and better tolerated in the stomach than aspirin (acetylsalicylate).

In the case of *Digitalis lanata,* two cardiotonic compounds are isolated from the leaves: digitoxin in large quantities and digoxin in small quantities. Only digoxin has interesting pharmaceutical properties, but it cannot be produced either chemically or by microbial bioconversion. A bioconversion of highly pharmaceutical interest is the 12-hydroxylation of the cardiotonic drug beta-methyl-digitoxin into the more desirable, less toxic drug, beta-methyl-digoxin, by cell cultures of *Digitalis lanata* (Ŗeinhard and Alfermann, 1980; Alfermann *et al.,* 1985). Using selection techniques, higher-yielding cell lines have been isolated and the most effective bioconversion achieved so far has been 1g/litre during a 28-day cultivation period (Heble *et al.,* Bio-Organic Division, Bhabha Atomic Research Centre, Trombay, Bombay, India).

4. Root cultures

After the pioneering work of White (1939) who established tomato root cultures capable of unlimited growth in media containing macro- and micronutrients, sucrose and yeast extract, Dawson, in 1942, succeeded in growing the roots of the tobacco plant. In 1957, Solt observed that the increase of nicotine concentrations in tobacco root cultures closely paralleled the increase in root tissue as measured through root length, number of branches and dry weight accumulation (in Flores *et al.,* 1987). It was also shown that root cultures of henbane (*Hyoscyamus niger*) synthesize hyoscyamine (in Flores *et al.,* 1987).

In *Datura* cells, alkaloid production was inversely correlated to the growth rate of callus cultures (Lindsey and Yeoman, 1983, in Flores *etal.,* 1987). Undifferentiated calli of *Atropa belladonna* do not produce the tropane alkaloid hyoscyamine but production does occur when roots form on the callus (Bhandary *et al.,* 1969, in Flores *et al.,* 1987). The cardiotonic glycosides of *Digitalis* are produced when calli are induced to undergo embryogenesis following treatment with plant growth regulators (Kuberski *et al.,* 1984, in Flores *et al.,* 1987). All these observations have led researchers to use continuously growing, organized plant tissue cultures or even cultures of organs such as roots for the production of secondary metabolites.

Chilton *et al.* (1982) discovered that the soil micro-organism, *Agrobacterium rhizogenes,* caused a "hairy root" disease affecting a wide range of dicotyledonous species and resulting in the proliferation of fast-growing adventitious roots at the host wound site (due to the stable integration of a portion of the Ri plasmid of *A. rhizogenes* into the plant genome). Hairy roots were then established as aseptic cultures after treatment with antibiotics. Flores and Filner (1985, cited in Flores *et al.,* 1987) were the first to demonstrate that "hairy roots" showed stable growth and produced secondary metabolites, namely the tropane alkaloids of *Hyoscyamus.* Researchers of the Plant Biotechnology Group, Agriculture and Food Research Council Institute of Food Research, Norwich Laboratory, United Kingdom, used transformed root cultures as a genetically and biochemically stable system for the study and production of secondary metabolites. The resulting stable high-producing lines may potentially be exploited directly as cultures in bioreactors or as plants in the field following plant regeneration (Rhodes *et al.,* 1988).

Axenic transformed root cultures of *Nicotiana rustica* and *Datura stramonium* (which respectively produce nicotine and the tropane alkaloids hyoscyamine and scopolamine) were developed following inoculation of plant material with *Agrobacterium rhizogeness* train LBA9402. The exact nature of the factors leading to root formation are still poorly understood. Transformed roots grow fast by plant standards; they show a logarithmic pattern of growth with doubling times which can be less than 48 hours and are characterized by a high degree of branching. This growth is associated with the production of the characteristic secondary metabolites that resemble the parent plant species in terms of both absolute amounts and spectrum of products (Rhodes *et al.,* 1988).

More than twenty hairy root clones of *Hyoscyamus muticus* (Flores and Filner, 1985, cited in Flores *et al.,* 1987) produced tropane alkaloids at the same concentrations as in the whole plant or in normal root cultures. Alkaloid production has been stable in two selected clones for over 40 monthly passages, but decreased markedly when roots were induced to form calli, and then reappeared when calli underwent root differentiation (Flores *et al.,* 1987). Furthermore, biomass production was very high: from an initial

inoculum of 2 to 4 mg (1 to 2 root tips), a typical hairy root clone of *Hyoscyamus muticus* grown in batch culture over three weeks showed a 2,500 to 5,000-fold increase, i.e. a higher increase than that obtained with the fastest cell suspensions. The results with *Hyoscyamus muticus* roots were extended to *Datura* and *Scopolia* species (Flores *et al.*, 1987).

It has been shown that hairy root cultures of *Beta vulgaris* and *Nicotiana rustica* synthesized betalains and nicotine plus anabasine, respectively, and that a significant portion of tobacco alkaloids was found in the growth medium of a 20-day batch culture. Hairy roots of *Atropa belladonna* were shown to form atropine and scopolamine at concentrations comparable with those found in field-grown plants and higher than those found in untransformed root cultures (Kadama *et al.*, 1986, in Flores *et al.*, 1987). Marro *et al.* (1986, in Flores *et al.*, 1987) examined 29 hairy root clones of *Scopolia japonica* and isolated two highly productive clones: clone S1 which accumulated scopolamine to 0.5% (w/w) dry weight and clone S22 which synthesized hyoscyamine at 1.3% (w/w) dry weight. Normal root cultures of several *Hyoscyamus* species accumulated hyoscyamine and scopolamine in the range of 0.04% to 1.1% (w/w) dry weight and 0.06% to 0.3% (w/w) dry weight, respectively (Hashimoto *et al.*, 1986, in Flores *et al.*, 1987).

The production of alkaloids derived from the tropane ring by root cultures, as well as that of beta-xanthine, is not exceptional. Hairy root cultures have been used to produce the secondary metabolites of the family Asteraceae (1,000 genera and over 15,000 species), i.e. the sesquiterpene lactones and the polyacetylenes. The latter are very active against bacteria, fungi and nematodes. They are found in the roots and their synthesis may be elicited by infection with fungal pathogens. During 1986, over 30 normal and hairy root clones from the genera *Ambrosia, Bidens, Rudbeckia* and *Tagetes* were established by Flores *et al.* (1987). These clones grew faster than their normal counterparts, produced thiophene-like compounds similar to those of normal roots and were stable for several monthly passages. It was concluded therefore that these organ cultures could synthesize polyacetylenes and their cyclic derivatives (Flores *et al.*, 1987).

According to results obtained at Cornell University, Ithaca, New York, cultures of onion roots in bioreactors are more favourable for the production of onion aroma than cell suspensions. These differentiated tissues are able to produce directly the complex chemical aromatic compounds that are then released into the culture medium and can be subsequently isolated by high-pressure liquid chromatography. Thirteen different species of onions from Europe, Japan and North America have been tested and several seem to be valuable for the industrial production of onion aroma. Culture conditions have been studied and it was reported that the final aroma could be modified by changing the precursors in the culture medium. The onion aroma produced by onion roots in culture would be much more related to the natural one than current available preparations (in *Biofutur,* no. 71, September 1988, p. 13).

Root cultures could thus become commercial sources of many secondary metabolites following the example from Rhodes *et al.* (1986, in Flores *et al.*, 1987) who reported the production of nicotine by hairy root cultures of *Nicotiana rustica* in a two-stage batch/continuous-flow system. These cultures showed a rapid growth rate and released a major portion of nicotine into the culture medium. A prototype for a large-scale bioreactor has now been developed for growing hairy roots of *Hyoscyamus muticus* (in Flores *et al.*, 1987).

Transformed roots are also being used to elucidate the control mechanisms of secondary metabolism pathways with a view to enhancing the production of metabolites. For instance, Rhodes *et al.* (1988) have been developing an approach to manipulate the metabolic pathway at the level of the individual gene and thus the individual enzyme. It involves identifying genes coding for enzymes which are under-expressed in culture, then to isolate the gene and to re-introduce it into the plant under the control of de-regulated, high-expression promoters to increase carbon flux through the pathway. In addition to putrescine methyl-transferase (PMT), ornithine decarboxylase (ODC) limits the part of the metabolic pathway common to nicotine and hyoscyamine biosynthesis. Thus, Rhodes *et al.* (1988) have transferred the gene coding for ODC from *Saccharomyces cerevisiae* into transformed roots of *Nicotiana rustica* where it is under the regulatory control of the cauliflower mosaic virus 35S promoter. The gene was integrated into the plant genome and was successfully transcribed into a fully active enzyme. The pattern of expression of other enzymes involved in biosynthesis of the alkaloids was unaffected in the transformed cells.

Another approach is to study the co-ordinated regulation of the expression of the entire pathway in order to understand how the expression of the group of genes is temporally and developmentally regulated. This would lead to the manipulation of this regulation to optimize expression of the whole pathway (Rhodes *et al.*, 1988).

To sum up, the use of root cultures as a commercial source of secondary metabolites will depend on the scaling-up of production and recovery techniques, on the further knowledge of the regulatory signals which induce the production of compounds at over 10% dry weight, and on the identification of high-value biochemicals in the roots (Sasson, 1988).

Industrial production of useful biochemicals by higher-plant cell cultures

1. Market-value estimations

Economic considerations govern the importance attached to the production of natural substances and biochemicals (see Table). The estimated annual market value of pharmaceutical products of plant origin in industrialized countries was over US $20 billion in the mid-1980s. The annual market value of codeine and of the anti-tumour alkaloids, vinblastine and vincristine, has been estimated at about US $100 million per product (Pétiard and Bariaud-Fontanel, 1987). The world-wide market value of aromas and fragrances has been estimated at over US $4 billion in 1980 and is expected to rise to US $6 billion in 1990 (Rajnchapel-Messai, 1988).

In 1988, the estimated annual market value of shikonin (for details see below) was about US $600,000 which is far from the US $20 to US $50 million investment of the original research and development work. However, the final cost of the product fell to US $4,000 per kg which compares with US $4,500 per kg for the substance extracted from the roots of *Lithospermum erythrorhizon*. It should be noted that Kanebo, the Japanese cosmetics corporation, which developed lipsticks containing shikonin, realized a turnover of about US $65 million over two years in Japan through the sale of 5 million lipsticks, each lipstick selling for US $13. In the Republic of Korea and China, Mitsui Petrochemicals Ltd today intends to market the product itself (Rajnchapel-Messai, 1988).

Economic data for some substances of plant origin

SUBSTANCE AND USE	ANNUAL NEEDS	INDUSTRIAL COST (US$ PER KG)	ESTIMATED ANNUAL MARKET VALUE (IN US$ MILLION)
Pharmacy			
ajmalicine	3- 5 tonnes	1,500	4.5-7.5
codeine	80-150 tonnes	650-900	52-135
digoxin	6 tonnes	3,000	18
diosgenin	200 tonnes	20-40	4-8
vinblastine			
vincristine	. 5- 10 kg	5 million	25-50
Food-additives and fragrances			
jasmine oil	100 kg	5,000	0.5
mint oil	3,000 tonnes	30	90
natural vanillin	30 tonnes	2,500	75
Cosmetics			
shikonin	150 kg	4,000	0.6

Sources: Fontanel, A.; Tabata, M. 1987. Production of secondary metabolites by plant tissue and cell cultures. Present aspects and prospects. *Nestlé Research News 1986-1987*, pp.93-103.

Rhodes, M.J.C.; Robins, R.J.; Hamill, J.; Parr, A.J. 1986. Potential for the production of biochemicals by plant cell cultures. *New Zealand Journal of Technology*, 2, pp.59-70.

Scragg, A. 1986. The potential of plant cell cultures in biotechnology. *IBL*, pp.44-7.

Rajnchapel-Messai (1988).

2. Major constraints of industrial production

Industrial or commercial scaling-up of the production of useful substances by cell cultures or suspensions should take into account the following properties, some of which could be major constraints. These are: slow growth of cells with doubling times of 24 to 48 hours requiring usually two to three weeks to provide sufficient biomass; susceptibility to microbial contamination; use of axenic cultures; oxygen needs; and susceptibility to shearing stresses due to the large cell size which on average is 200,000 times larger than

that of bacteria. In general, cell multiplication and metabolite synthesis are uncoupled, the latter occurring at the end of the growth phase.

3. Achievements

Significant progress has been made to overcome the major constraints of industrial production since the late 1960s. Heble *et al.* of the Bio-Organic Division of the Bhabha Atomic Research Centre, Trombay, Bombay, India, are carrying out research on mass cultivation in 20-litre capacity bioreactors of selected cell lines of *Rauwolfia serpentina* (ajmaline, reserpine), *Papaver somniferum* (thebaine, codeine, morphine), *Artemisia annua* (artemisinin), and other plant species (Chadha *et al.*, 1988).

In Japan, the Nitto Denki corporation uses a non-continuous process for biomass production of *Panax ginseng* in 20,000-litre bioreactors, the yield being 500 mg dry matter per litre per day (Rajnchapel-Messai, 1988). Hashimoto *et al.* (1982, in Pétiard and Bariaud-Fontanel, 1987) succeeded in cultivating tobacco cells in 20,000-litre bioreactors for over two months with yields being 5.82 g dry matter per litre per day. Fujita *et al.* (1982) selected a highly productive cell line of *Lithospermum erythrorhizon* (Ko-shikon), and developed a two-stage growth and production method for shikonin which is an anthraquinone extensively used in Japan for its anti-inflammatory and antibacterial properties and also as a dye. This substance is found in the roots of the plant which accumulates up to 2 to 3% of its dry weight as shikonin, the plant taking 5 to 7 years to reach a size useful for commercial production. No method is yet available to synthezise shikonin. In 1983, Mitsui Petrochemical Industries Ltd reported a technique for the industrial-scale production of shikonin by cell cultures. The process involved two stages: (a)plant cells were first grown in a 200-litre capacity bioreactor and (b)the resulting biomass was then transferred into a second bioreactor in which the composition of the culture medium favoured the synthesis of shikonin. Even though the capacity of the second bioreactor was only 750 litres, the Japanese technique marked an important turning point in the bio-industrial application of plant tissue cultures (Fowler, 1984b). In a 23-day culture period, cells grown in the 750-litre bioreactor accumulated 23% of their dry weight as shikonin (Curtin, 1983). The productivity of *L. erythrorhizon* cell cultures was 60 mg per g of cells per week, that is 1,000 times higher than that of the plant roots which required a longer time period of 5 to 7 years.

The success of shikonin production was due to the selection of a cell line which accumulated a ten-fold higher level of shikonin than that found in roots of the mature plant. This achievement resulted from an empirical, labour-intensive search for optimal growth conditions and production media which was further facilitated by a visual selection for overproducing cells. Cell cultures have now thus become the major commercial source of shikonin (Flores *et al.*, 1987).

In addition to the work on shikonin, Japanese scientists were able to obtain higher quantities of berberine from growing cells of *Coptis berberica.* This plant species accumulates significant amounts of berberine in its roots in four to six years; similar concentrations could be obtained in four weeks using tissue culture. Hara *et al.* of Mitsui Petrochemical Industries Ltd have isolated a cell line of *Coptis japonica* that contains 10% of berberine (dry weight) and which could produce about 1,500 mg of this antibacterial and antipyretic alkaloid per litre in 14 days. Analysis of cell lines by flow cytometry indicates that the increase in berberine production resulting from cell selection is related to the increase of the number of cells with a high content of berberine, rather than to an overall increase of this content in all the cells. Thus, the high-yielding line is most probably heterogeneous (in *Biofutur,* no.83, October 1989, p.17). Industrial production of geraniol is also being developed by the cosmetic company Kanebo (Rajnchapel-Messai, 1988).

In the Federal Republic of Germany, Alfermann *et al.* (1985) of Boehringer Mannheim AG were able to grow cells of *Digitalis lanata* in 200-litre bioreactors and obtain 500 g of beta-methyl-digoxin in three months; the bioconversion rate of beta-methyl-digitoxin was very high, up to 93.5%, if the non-used substrate was recycled. Ulbrich *et al.* (1985) cultured *Coleus blumei* cells in a 42-litre bioreactor fitted with the module spiral stirrer; using this system with aeration, they reported high yields of rosmarinic acid (5.5 g per litre), representing 21% dry weight of cells. Heble and Chadha (1985a, b) reported the successful cultivation of *Catharanthus roseus* cells in 7 to 20-litre capacity bioreactors, modified to provide air lift and agitation, in single and multiple stages. The cells produced high levels of total alkaloids comprising ajmalicine and serpentine as the major components. It was shown that plant cells could withstand shear to some extent, and that judicious use of air lift and low agitation was advantageous. Researchers at Ciba-Geigy AG, Basel, Switzerland, have produced the alkaloid scopolamine from cell cultures of *Hyoscyamus aegypticus* grown in air-lift bioreactors (in *McGraw-Hill's Biotechnology Newswatch,* 21 January 1984).

Sanguinarine is a benzophenantridin alkaloid extracted from the roots of *Papaver somniferum.* Three to four years are needed for plant maturation before the substance can be extracted. Cell cultures have been used to produce large quantities of this alkaloid, which is used in toothpastes and mouth lotions to combat dental plaque and tooth decay. Commercial production will be the result of a joint venture between the Plant Biotechnology Institute of the National Research

Council of Canada, Saskatoon, and Vipont Research Laboratories Inc., Fort Collins, Colorado, USA. The manufacturing process will use the eliciting power of extracts of a fungus, an unspecified *Botrytis*, that induce the synthesis of sanguinarine and dihydrosanguinarine by plant cells. Pilot experiments have shown that in semi-continuous cultures, which could be elicited twice, the production rate of alkaloids reached 3% of dry biomass (in *Biofutur*, no.79, May 1989, p.13).

These examples and others show that industrial production of plant cell biomass and secondary metabolites is possible with equipment and processes analogous to those used with micro-organisms. From the economic viewpoint, Zenk made an estimate of US $500/kg for the production of a drug by cell cultures at a rate of 1 g per litre. Goldstein *et al.* (1980) of Miles Laboratories Inc. (Bayer AG) also analyzed the economics of plant-cell culture methods and suggested that products costing more than US $1,000/kg were suitable. With higher-yielding cultures, the production cost could be much lower. For a 10% dry weight yielding product, the cost could come down to US $228/kg. This could be further reduced if immobilized cells were used (Sahai and Knuth, 1985; Heble and Chadha, 1986). According to a study carried out in Japan, any substance of plant origin with a value exceeding or equal to US $80/g could be profitably produced by cell or tissue cultures. This would even apply to substances of which the retail price varies between US $250 and US $500/kg, i.e. many raw pharmaceutical products, aromatic compounds, condiments and fragrances (Fowler, 1984b; Vasil, 1987, 1988).

Immobilizing cells in a gel, which is permeable to the molecules of the nutrient medium or on polymers (with a view to preserving their metabolic capacity and to using them several times), has the advantage of extending the production time of cells (over six months) and of making the cells catalyse the same reaction almost indefinitely. Active research has been carried out in this area since the early 1980s. Thus, the team of Furusaki of the Department of Engineering, Tokyo University, has developed a bioreactor for the production of codeine, which is mainly used as an anti-cough compound in pharmacy. Poppy cells have been immobilized in calcium alginate beads and they catalyze the conversion of codeinone into codeine. This technique enabled the Japanese researchers to overcome the drawbacks of plant-cell bioreactors arising from the instability of cells and the low yields of the desired compounds. They decreased the size of cell clusters (2.5 mm in diameter), thereby increasing their life-span and obtaining yields of codeine that were equal to those in non-immobilized cells (*in Biofutur*, no. 79, May 1989, p.14).

The use of immobilized cells should bypass the direct extraction of the compounds from the biomass as the products now arise in the medium itself. Examples of this approach include the production of caffeine, capsaicin and berberine. Many metabolites however still appear to accumulate in the cell vacuoles and it is therefore important to further gain information on how these metabolites may be made to diffuse out into the culture medium. Although immobilized cell technology is a promising technique, especially aimed at decreasing production costs, clearcut examples still do not exist which would demonstrate a gain of productivity on an industrial scale (Pétiard and Bariaud-Fontanel, 1987).

4. Prospects

With the onset of the 1990s, only Japan and, to a lesser extent, the Federal Republic of Germany are really engaged in the industrial production of secondary metabolites by plant cell cultures. The only marketed product (as of 1990) remains shikonin. In Japan, seven private corporations have created a common subsidiary in research and development on plant cell cultures. The Plant Cell Culture Technology (PCC Technology) has been set up with the support of the Japan Key Technology Centre (JKTC) by Kyowa Hakko Kogyo Co., Mitsui Petrochemical Industries Ltd, Mitsui Toatsu Chemical Inc., Hitachi Ltd, Suntory Ltd, Toa Nenryo Kogyo Co. and Kirin Breweries Co. Ltd. By contrast, most North American and European companies are not enthusiastic about the prospects regarding profitable industrial production (Rajnchapel-Messai, 1988).

Several factors or constraints could explain this situation. Firstly, the time needed for selection and stabilization of cell lines is about two to three years due to the difficulty of controlling and directing somaclonal variation. Consequently, there is a need for tedious and time-consuming screening of a large number of lines. Secondly, the lack of knowledge concerning biosynthetic pathways of secondary metabolites explains certain failures. For instance, it has not been possible to isolate a cell line with a good level of production of dimeric alkaloids of *Catharanthus roseus*, although these alkaloids are present in minute quantities in the plant (one tonne of dry leaves yields 0.5 to 2 g of these compounds). Thirdly, the difficulties of extraction of the desired compounds are serious, especially when the compounds accumulate as combined substances; this probably explains why the French company Sanofi-Elf-Bio-industries has abandoned the production of codeine and morphine by cells of *Papaver somniferum*. However, according to Steck of Sanofi-Elf-Bio-industries, technical and scientific difficulties can be overcome in fairly short periods resulting in lower industrial production costs. It is, though, essential that a worthwhile target be identified in order to secure the necessary investments (Rajnchapel-Messai, 1988).

Thus the real difficulties are identification of target compounds as well as economic and legal considerations. Production of secondary metabolites by

plant cell or tissue cultures must, of course, be competitive with other conventional means of production such as extraction from the field-grown plants, alternative enzymatic processes, chemical synthesis or semi-synthesis, microbial fermentation or improvement of the plant itself through somaclonal variation, genetic engineering, etc., followed by the regeneration of the plant from *in-vitro* cultures.

Production by cell cultures could be justified, however, for rare products that are costly and difficult to obtain through other means. According to Pétiard of the French company Francereco, this approach would be feasible only for products whose world annual potential market would be US $20 to US$50 million, with a minimum selling price of about US $400 to US-$500 per kg. Thus, ajmalicine and jasmine oil, in spite of their high selling price - US $1,500 and US $5,000 per kg, respectively - are not attractive for industrial production by cell cultures because of the size of their market, i.e. US $8 million for ajmalicine and only US $500,000 for jasmine oil, which would not permit the amortization of the investments to be made in research and development. On the other hand, mint aroma, which represents an annual market of US $90 million, has a selling price which is too low - US $30 per kg - to be worth lowering further through cell-culture production. The challenge lies therefore with the identification of economically profitable targets (Rajnchapel-Messai, 1988). But as many companies throughout the world have considered these aspects, it may be concluded that such target compounds that may have been identified will remain closely guarded secrets for some time to come.

In the pharmaceutical area, the number of economically-profitable targets also appears to be rather limited. Many plant substances - or their derivatives from semi-synthesis - are used in drugs, and their production cost is often very high; their individual economic weight is nevertheless rather low. They are used in small quantities in a medicine whose final price is due more to the considerable investments in research and development than to the cost of raw materials. Furthermore, the "plant production" approach is open to the strong competition of chemical synthesis that is very efficient in developing new molecules with highly specific activity. Thus, the development of new synthetic cardiotonic compounds in the USA has resulted in the decreased demand for *Digitalis* cardiotonic glycosides. It is also one of the causes for the non-commercialization of beta-methyl-digoxin produced by *Digitalis* cell cultures (Rajnchapel-Messai, 1988). However, commercial production was envisaged for vincristine and vinblastine from *Catharanthus roseus* cell cultures by Eli Lilly & Co., ubiquinone from *Nicotiana* (Matsumoto et al., 1982), L-dopa from *Mucunna pruriens* (Wichers and Pras, 1984) and digoxin from *Digitalis lanata* (Alfermann et al., 1985).

Prospects are more promising for the production of aromatic substances, flavouring compounds and food additives, and basic materials for fragrances by plant cell or tissue cultures. These products, although less valuable than pharmaceuticals, have larger markets. This explains the recent re-orientation of research, as is witnessed by the four-fold multiplication of the number of publications devoted to food additives and cosmetics that were submitted to the 1986 Congress of the International Association of Plant Tissue Culture, held in Minneapolis, USA.

In this area, however, the difficulties relate to the very low investments made in research and development by the relevant companies involved with agriculture, food and perfumeries. In addition, technical difficulties should not be underestimated because most aromas and fragrances, with a few exceptions such as vanillin and irone (which is a violet fragrance extracted from iris rhizomes that needs to be stored for one to three years) are mixtures of a large number of compounds, some of which are present in minute amounts but which are nevertheless vital for the final product to be accepted. It is therefore very difficult to reproduce such mixtures exactly by cell cultures. The evaluation of productive cell lines usually must rely on the smell and taste of the perfumer or aromatician thus necessitating that such work be carried out in locations having easy access to such people. Consequently it is almost impossible to conduct such work outside industrial companies. These companies, however, are usually very conservative in their outlook and with only one or two exceptions have usually set their face against the feasibility of producing aromas, fragrances and flavours by anything other than conventional means. At first sight, this appears to be a very narrow approach but it should be remembered that many of these companies rely on suppliers and growers for a number of their products. To seek to produce one product by alternative means (i.e. plant cell culture) could jeopardize the future supply of a number of other products simply by the supplier or grower now refusing to do further business. Thus the balance between producer and seller is a subtle one and is one which neither party wishes to see upset.

Other issues are: what will be the legal status of these new products? Will they be labelled 'natural'? Would it be possible to market them without a complementary toxicological study, as is currently the case for all substances extracted from new plant varieties bred and cultivated with conventional methods? The modification of the genetic heritage of these varieties is, however, much greater than the changes which may occur in cell suspensions (Pétiard and Bariaud-Fontanel, 1987).

Industrial production of food additives and fragrances

Food additives contribute to making foodstuffs palatable and attractive by enhancing or improving their flavour, colour and texture. Food technologies try to respond to these criteria especially with regard to the texture, taste and aroma of the foodstuff. The need to have the same taste and aroma in a specific foodstuff in order to suit the consumers' tastes makes it compulsory to use natural or artificial aromas, especially in certain products where they may be indispensable. A few decades ago, aromas were extracted from plant raw materials transformed by fermentation, grinding and heating. Later, chemical compounds, pure or as mixtures, completed the range of plant aromas. Nowadays, new compounds are added to the existing ones and they usually originate from enzymatic reactions, or are produced by biotechnological methods, i.e. by microbial and plant cells (Spinnler, 1989). Fermentation techniques are preferred to chemical synthesis if they are more cost-effective, e.g. in the production of citric acid and monosodium glutamate. Microbial synthesis is also the only means to produce the thickening additive xanthan.

Since the late 1950s, many food additives have been questioned mainly by national and international regulatory authorities, about their safety for long-term use and consumption. At the same time, the associations of consumers, aware of the inclusion of additives in foodstuffs, have been exerting pressure on governmental bodies to have chemical or artificial additives replaced by "natural" additives from plant or animal tissues, or be synthesized by micro-organisms or plant cell cultures. New production processes would allow food industries to respond to the favourable opinion about natural aromas and also to overcome the present constraints related to the climatic and political vagaries of supply in the producing countries and to take advantage of the difference in price between naturally- and chemically-produced compounds. For instance, in the case of vanillin, its price is about 20,000 FF per kg if it is extracted from the vanilla pod, whereas it costs only 50 FF per kg if it is produced by chemical synthesis from lignin. Although this difference is considerable, it is not possible to differentiate the two kinds of vanillin molecules through chemical or physical analyses. Hopefully for aromas with a restricted market, eventual fraud will be easily detectable and presumably will be rigourously monitored by those industrialists who will benefit most from conserving their existing sales (Spinnler, 1989).

Pigments are also food additives but their use has been strongly criticized by the associations of consumers in the 1970s, because most of them are produced by chemical synthesis and are unrelated to any naturally occurring material. In the European Economic Community, 24 pigments are currently authorized of which 10 are not of natural origin. It is true that the colour of foodstuffs is associated with their acceptance and the pleasure they procure. Thus, the list of pigments used for this purpose is bound to become longer but the trend is to replace artificial colourings by natural ones. Market forces however will determine whether the public will pay increased costs for "natural" products. The biotechnological methods used for the purpose of producing natural food colorants consist of growing micro-organisms, micro-algae and higher-plant cells (Langley-Danysz, 1987).

Extraction of pigments from plants is an old technique, e.g. anthocyanins from red berries, betanine from beet and curcumine from saffron. Some pigments cannot be extracted before the plant reaches the adult stage. This is the case of rocou, a pigment extracted from the seeds of a shrub, or of shikonin, extracted from the roots of *Lithospermum erythrorhizon.* In the case of shikonin, plant cell cultures have been very successful in the industrial production of this dye (see above).

1. Aromas and fragrances

Natural aromas are a mixture of numerous compounds: more than 500 have been identified in roasted coffee beans and 200 in apple. Natural aromas are susceptible to the conservation processes of foodstuffs such as sterilization, pasteurization, freezing, etc. Some aromas are altered by enzymatic or chemical reactions and usually disappear if stored for a long period. This is why substitutes have been sought for them since the end of the 19th century. Artificial aromas were manufactured from coal or oil derivatives and were added in very low concentrations (10-6 or ppm, and 10-9 or ppb). The present trends are either to produce synthetic molecules, which are identical to natural molecules, or to use biotechnological methods. The first category of aromas have the advantage of a constant composition, of not depending on the season and of being manufactured in such a way that the production is adapted to the market. Their drawback is of not being mixtures of substances that resemble the natural ones. This is why the biotechnological routes are now being increasingly preferred (Langley-Danysz, 1987). For instance, Dziezak (1986, in Langley-Danysz, 1987) reported that the characteristic aromas of cocoa and coffee have been produced by cell cultures of *Theobroma cacao* and *Coffea arabica,* respectively.

The value of the world market for aromas and fragrances was estimated at about US $3 billion in 1988 (Vaisman, 1988). In 1987, the ten first world leading companies represented more than two-thirds of the total annual turnover, whereas in 1981 the same proportion was attributed to the first 20 companies (Coërs, 1989):

	1987
International Flavors and Fragrances (IFF, USA) US$	746 M
Quest (Unilever NV, UK and Netherlands)	635
Givaudan (Hoffmann-La Roche AG, Switzerland)	462
Takasago Perfumery (Japan)	441
Firmenich (Federal Republic of Germany)	333
Haarmann and Reimer (Bayer AG, FRG)	327
Dragoco	250
Roure (Hoffmann-La Roche AG, Switzerland)	125
FDO (BASF AG, Federal Republic of Germany)	120
PFW (Hercules, USA)	100
Mane (France)	83
Robertet (France)	72
Sanofi-Elf-Bio-industries (France)	66
Florasynth (France)	-
T. Hasegawa Flavor Co. (Japan)	-
Ogawa (Japan)	-

According to the conclusions of a study carried out in France by a consulting firm, Précepta, the market of aromas and fragrances is soaring, compared with that of foodstuffs in general which is progressing at a slower pace. In the aroma and fragrance sector, 5% to 6%, and sometimes up to 12% of the total turnover, is devoted to research and development, i.e. one order of magnitude more than in the food sector (Coërs, 1989).

In Japan, the growth of the aroma and fragrance market was rather slow during the 1970s but the increasing consumption of perfumed home products ("air purifiers") induced a more rapid growth rate of the market which was evaluated at about US$0.8 billion in 1988. Several industrial companies have emphasized their diversification efforts in this sector: Takasago Perfumery, T. Hasegawa Flavor Co., Soda Aromatic Co., Shiono Koryo Kaisha Ltd, and Ogawa. Thus, Soda Aromatic Co. controls the market of deodorant substances and 80% of the Japanese market related to the deodorization of natural gas (Vaisman, 1988).

Plant cell cultures are a promising means of production of aromas and fragrances in Japan (Vaisman, 1988). Thus, Kirin Breweries Co. Ltd, Kyowa Hakko Kogyo Co., Mitsui Toatsu Chemical Inc., Mitsui Petrochemical Industries Ltd, Hitachi Ltd and Toa Nenryo Kogyo Co. have concluded co-operative agreements to produce aromatic substances by plant cell cultures. The expertise of Kirin Breweries in the manufacture of fragrances from humulane, a by-product of the processing of hops, and that of Mitsui Petrochemical Industries Ltd in the large-scale production of shikonin are major contributions to the success of this association. Ajinomoto Co. has also filed a patent on the production of safranal (which gives saffron its spicy taste) by cultures of cells from the pistil of *Crocus sativus* (similar research work was carried out in the laboratory of Yeoman in Edinburgh). Kuraray Co. is currently producing lavandol by cultures of lavender cells, while researchers at Kitasato University are using *Eucalyptus* cell cultures to catalyze the bioconversion of alpha-menthol (Vaisman, 1988). All these products are promoted by emphasizing their natural origin to counteract the consumers' reluctance to use artificial products.

In France, the consumption of medicinal and aromatic plants has trebled in the past 20 years: a quarter of the production being utilised as raw materials (flavourings and infusions or steeping of herbs), and three quarters being processed for the pharmaceutical, food, fragrance and cosmetics industries. In 1986, consumption of these plants in France reached 30,000 tonnes. Domestic production was only 13,000 tonnes, the balance being achieved by importation. The trade deficit was valued at 272 million FF (Comar, 1988).

Initiatives have therefore been taken to promote the French production of medicinal and aromatic plants, while ensuring better protection of the consumers. This has involved direct help to the farmers to enable them to respond to the increasing demand and also to achieve better post-harvest operations. Another initiative is that of a new biotechnology company, Biophytec, established in Lyon, which aims to apply biotechnologies to the production of medicinal and aromatic plants, with a view to marketing products with medicinal properties and food additives. Finally, efforts have been made to obtain accelerated authorization to market certain plants or plant extracts having medicinal properties (Comar, 1988).

The world leading pharmaceutical groups, such as BayerAG, BASFAG and Hoffmann-La Roche AG, have been increasingly involved in this sector and have

bought many smaller companies. In France, Sanofi-Elf-Bio-industries has made large investments, whereas agrobusiness companies such as Pernod-Ricard or chemical corporations such as Unilever NV and Procter & Gamble Inc., have been trying to integrate this new activity into their conventional ones. Other potential participants are the petrochemical companies such as Esso and Royal Dutch-Shell (Coërs, 1989).

This new situation will limit the negotiating capacity of small firms which are mainly involved in the processing of natural products. As they have been slow to diversify their activities, they now must make a choice between necessary growth and confrontation with more powerful companies. Old corporations that supply the industry with the natural raw materials have to face the competition of biotechnological products; the only hope for them is to concentrate their activities increasingly on the supply of high-value added products (Coërs, 1989).

However, the future role of the small- and medium-size companies in the sector of aromas and fragrances could be analogous to that of small biotechnology companies which concluded agreements with the large and powerful groups, e.g. the British company, Imperial Biotechnology, which has research agreements with Nestlé, Tate & Lyle, and Beecham plc (merged in 1989 with the American pharmaceutical corporation Smithkline Beckman into the new group Smithbee). If the biotechnological production of aromas and fragrances proves cost effective, the small companies which have mastered the relevant biotechnologies will become the natural partners of the large food, chemical and pharmaceutical groups. Such new ventures or partnerships will be promoted by the trend which makes the frontiers between aromas, fragrances, pharmaceuticals, medicines and biochemicals progressively disappear. This is, for instance, substantiated by the research programmes of Nestlé which link together activities in nutrition, health and dietetics. An area which seems promising and which can be a driving factor for the industrial production of fragrances, including by plant cell cultures, is that of aromachology.

2. Aromachology: new prospects for fragrance production and commercialization

Since the early 1980s, Shiseido, the leading Japanese cosmetics company, and other manufacturers of flavours and fragrances have been carrying out research and experiments in a new area named aromachology, not to be confused with aromatherapy which uses essential oils for therapeutic purposes. This new science aims at studying the effects of scents and fragrances on the physical and mental conditions of human beings, and at using these substances to modify these conditions. In 1989, in co-operation with Kajima Construction Company, Shiseido was testing the effect on working conditions of the release of a specific fragrance through the air-conditioning system into the offices at certain hours of the day. The objective, as stated by Shiseido, was to try to eliminate the stress due to work and to improve the effectiveness and productivity of employees. Other similar experiments are being conducted by Shiseido with another civil engineering company, Ujima, and with Takasuna and Shimizu Construction Company. Furthermore, at the 1989 automobile show in Tokyo, a prototype car was displayed with an air-conditioning system which releases a jasmine fragrance to keep the driver awake (Leventer, 1990).

In 1984, the world leading company International Flavors and Fragrances (IFF) filed the first patent on 'a method which induces the decrease of physiological and/or subjective reactions to stress in human beings'. But it was only in 1988-1989 that the American company could master a method to evaluate in quantitative terms and in a reliable way the changes of humour and mental state. This method combines physiological measures such as heart rhythm, blood pressure and brain waves with the filling in of questionnaires such as those used in psychiatry to evaluate the effects of medicines. According to the statements made by the executive in charge of technical development at IFF, the statistical results obtained are considered to be satisfactory and can support the effectiveness of the products tested. However, there is a placebo effect of about 60% which IFF has, of course, tried to reduce (Leventer, 1990). The Japanese researchers are also using the variations of physical indicators as well as encephalographic tests (measures of alpha- and beta-waves). According to Takasago Perfumery, a manufacturer of aromas, the methodology is considered to be valid and it has been developed in co-operation with Japanese universities and scientific institutions (Leventer, 1990).

New products with specific effects are to be marketed. In 1984, Shiseido marketed two Cologne-water perfumes for men, 'Because Psyche Refresh' and 'Because Psyche Cool'. The Japanese company asserts that these are the first products in the world whose stimulating or sedative effects have been established scientifically. In 1988-1989, the same company developed with Hattori Seiko and marketed an automatic alarm clock which releases before ringing a perfume made of pine and eucalyptus fragrances which should stimulate the body. Kanebo, the second Japanese cosmetics company, developed a fragrance containing lavender, camomila, anise, among others, which has sedative and sleeping effects. Matsushita Electric sells cards, which release sedative or stimulating fragrances when they are introduced into small 'bread toasters'. Kanebo is now marketing handkerchieves with microcapsules interspersed between the textile fibres which release the fragrance when used (Leventer, 1990).

Cosmetic products with the same objectives as those produced by IFF have also been marketed by Avon in co-operation with Takasago Perfumery, and by the French world leading cosmetics company, L'Oréal. Although the companies that are investing in aromachology claim that they do not wish to enter into the medical research area and that for the time being they are focussing their efforts on relaxing or stimulating compounds, it can be assumed that this is a promising area not only for those companies which manufacture fragrances but also for those which would like to seize this opportunity to widen their share of the fragrance market. It might not be unusual that in the future people buy a perfume not only because of its scent but also because of its relaxing, stimulating or physiologically-specific effect.

In this respect, it is worth mentioning the report made jointly by the Fragrance Foundation, IFF and the University of Cincinnati about their discovery of fragrances that can increase vigilance. Elsewhere, at Duke University, research is being carried out on the effect of fragrances on violence. At the University of Tsukuba, in collaboration with Shiseido, rates of recovery of athletes after strenuous activity are being monitored using certain fragrances to be breathed in before or after the exercise (Vaisman, 1988; Leventer, 1990).

Very little information has yet been reported on the reaction of the general public to these developments. Will they accept the well-being afforded by the new products, or will consumers' associations, trade unions and public bodies in charge of health regulations react to the potential side-effects of the products on physical and mental health?

Conclusions

One should not conclude hastily that cell cultures have no future in the area of production of useful substances. Many laboratories carry out their research and development programmes because the possibilities exist to increase the yields of their production processes. Furthermore, even if certain compounds are not worth developing up to a commercial stage, some biosynthetic compounds could be used as valuable precursors for organic synthesis, or could themselves constitute entirely new products. The market size for the end product, the cost/benefit ratio of the production technology, the competition with substitutes and the existence of other sources of supply are major factors which influence the choice of the appropriate manufacturing technique, especially when deciding in favour of plant cell or tissue cultures. Thus, it is probable that metabolites, synthesized through simple enzymatic reactions under the control of a single gene, could be more efficiently produced by genetically-engineered microbial cells rather than by plant cell or tissue cultures (in United States Office of Technology Assessment, OTA, 1983). Finally, it is not easy to identify economically profitable targets because of industrial secrecy or confidentiality which prevails in this area and because of the infrequent contacts between researchers and companies working in the field of aromatic and medicinal plants (Rajnchapel-Messai, 1988).

Japanese scientists and companies however are rather optimistic for the future of plant cell culture and they are probably right. In 1989, the Japanese Ministry of Agriculture, Fisheries and Forestry launched, through their Forestry Agency, a new programme named 'Green Spirit Project'. With a budget of about 110 million yen, this project aims at producing essential oils, resins and glycosides out of plant residues (wood, branches, bark, leaves). Potential applications of the compounds to be produced are food additives (in particular sweeteners), medicines and fragrances (in Biofutur, no. 79, May 1989, p.8).

In Europe, Canada and the USA, a number of companies do carry out research in this area and closely monitor current progress elsewhere. For instance, in France, Sanofi-Elf-Bio-industries maintains a reduced research activity in this area and has supported the research work of Chénieux and Rideau on the production of bioconversion of ellipticine - an anti-tumour alkaloid - by cell cultures of *Ochrosia elliptica* (Apocynaceae). The same group is involved through the Mero Company in the production of fragrance compounds (flower and fruit) by Ambid and his co-workers at the National School of Agriculture of Toulouse. Nestlé's subsidiary, Francereco, carries on its research programme on the production of metabolites by plant cells and has established fruitful co-operation with many university teams such as that of Cosson at the Faculty of Pharmacy, Chatenay-Malabry, in the south of Paris. An industrial project awaits a decision that will be taken on economic grounds rather than on scientific or technical ones. The pharmaceutical company, Roussel-Uclaf, is interested in the work carried out at the University of Montpellier on the production of diosgenin. When the French chemical company Rhône-Poulenc bought the German phytopharmaceutical firm Nattermann, it dissolved the plant cell-culture project group and its laboratory - one of the largest plant cell-culture laboratories in the world - in late 1988. Rhône-Poulenc is nevertheless looking for an appropriate policy in the field of plant cell cultures (Rajnchapel-Messai, 1988).

At the European level, all these issues were discussed at the Symposium on 'Bioproduction of metabolites by plant cell cultures', held in Paris in September 1988 and organized by the International Association of Plant Tissue Culture and the French Association pour la Promotion Industrie-Agriculture (APRIA, Association for the Promotion of Industry-Agriculture). The organizers expected that at least two or three targets could be identified in order to launch a research programme at the European level and that

government support would be forthcoming in an area where financial risk is important, but where profits could be large.

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