KIMIKA 8: 49-70 1992)
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Review

Plant secondary metabolites by tissue culture

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Introduction

Since time immemorial, man has depended on compounds extracted from plants for a wide variety of applications, ranging from drugs, to food colorants and additives, and dyes. These compounds comprise a variety of structures and are broadly classified as **secondary metabolites** -- compounds which plants synthesize for a variety of support functions not directly involved with the fundamental life processes of photosynthesis, respiration, or growth.

While advances in chemical synthetic techniques have lessened the extent of man's direct dependence on plant extracts, they continue to play an important role. For example, plants still provide active constituents in about 25% of prescribed medicines and are now being studied for new compounds to cure cancer and AIDS. Plants are still used as natural colorants and food additives. It is not likely that synthetics will replace these natural products in many of its applications (1).

Phytochemistry, the systematic study of plant secondary metabolites, groups these compounds according to their biosynthetic origins in the plant. There is a rough relationship between the biosynthetic origin of metabolites and their chemical structure (Figure 1). According to this scheme, we can map out the biosynthetic relationships of the many thousands of compounds found in plants.

Over the past 20 years, the phytochemical approach has been augmented by the development of new techniques and discoveries from biochemistry, plant physiology and molecular biology; the methodology common to these is plant tissue culture.

Plant tissue culture invplves the maintenance of live plant cells under controlled aseptic conditions. The tissues can be **undifferentiated** (individual cells or a mass of cells, called a

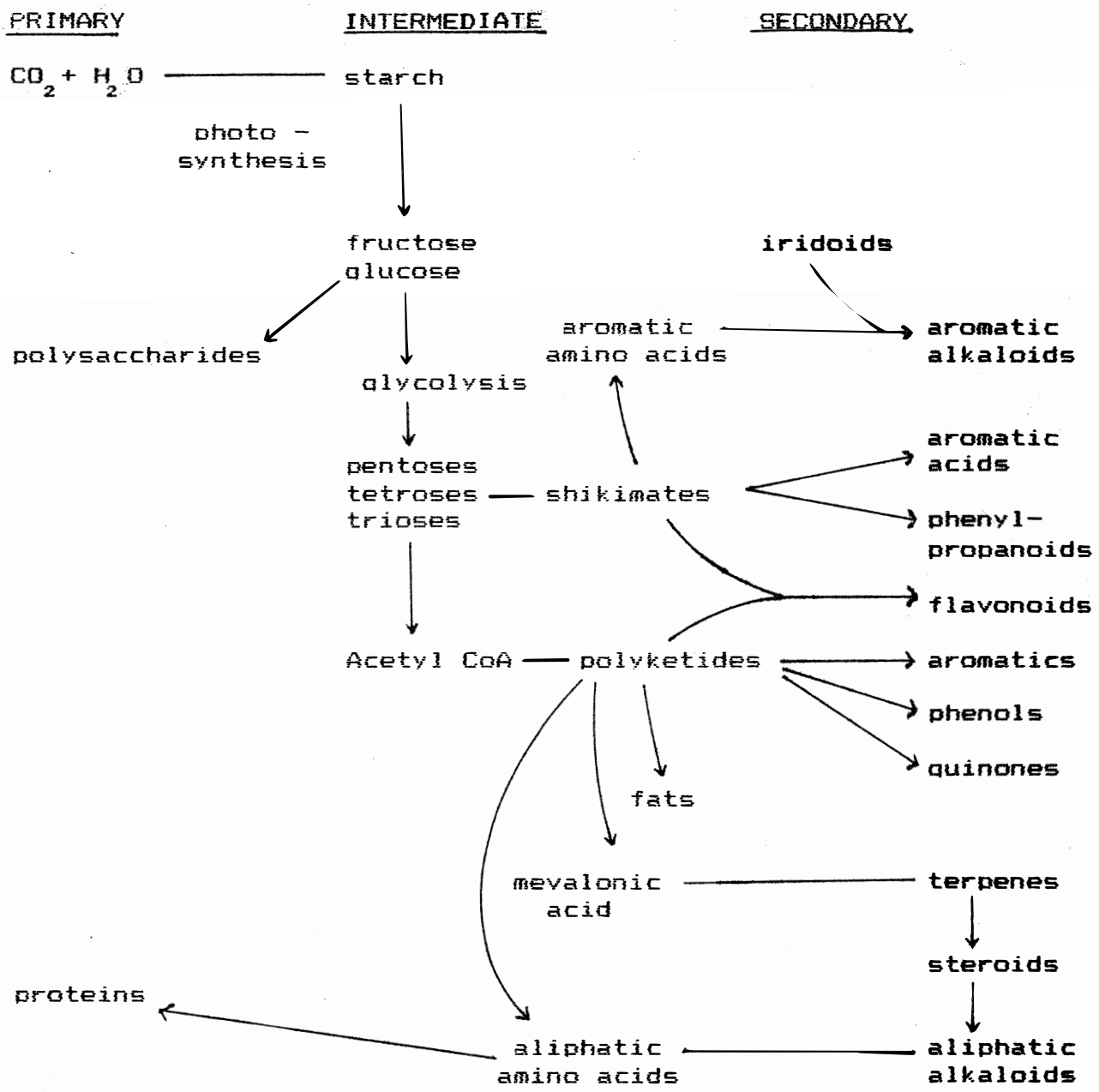


Figure 1. Scheme of biosynthetic relationships of plant secondary metabolites.

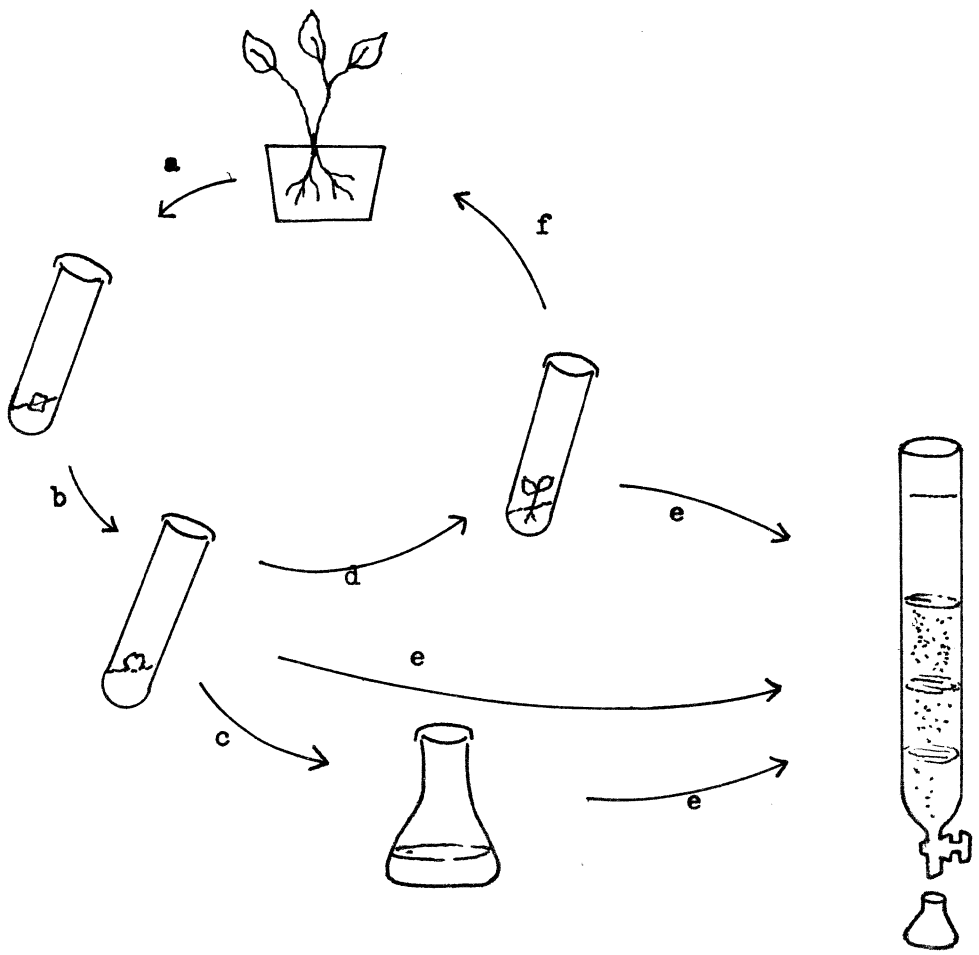


Figure 2. Outline of operations in tissue culture. a. Explant is placed on growth medium. b. Callus forms after 5 to 10 days. c. Suspension culture is used for more efficient mass formation or biosynthesis of metabolites. d. Callus can be allowed to differentiate under appropriate conditons. e. Secondary metabolites can be isolated from tissue culture suspension culture or from differentiated tissue. f. Whole plants can be regenerated.

callus) or **differentiated** into the various parts (roots, shoots, etc.). Figure 2 outlines the various operations involved in the culture of the plant tissue and the purification of the secondary metabolites from the culture. In principle, the metabolites can be isolated from the callus, suspension culture, or differentiating plant tissue. This strategy differs from that undertaken by biologists for the selection and propagation of plants.

Plant tissue culture was first explored by biologists around the turn of the century as a technique for plant breeding and propagation (2); during the 1960's, the technique was applied by chemists to the study and production of secondary metabolites (3).

Today, the use of plant tissue culture has opened up new opportunities for the chemical study of plant metabolites. There are many reasons for the great interest in plant tissue culture. From a scientific viewpoint, it gives important access to the study of the biochemical mechanisms occurring in the plant cell. From a commercial production viewpoint, it enables independence from seasonal variations, pests, diseases and from uncertainties of plant supply and greater control of quality and quantity.

The objective of this review is to briefly describe recent developments in plant tissue culture and to highlight the power of this technology. It is important to realize that the greatest advances are likely to arise from a multidisciplinary approach involving phytochemistry, biochemistry, molecular biology, and plant physiology, and other related disciplines.

Current status

The use of plant tissue culture for the production of secondary metabolites is now about 30 years old. The pace of research in this field has been one of cycles of great optimism and reserved enthusiasm. Until recently, research in this area was limited to empirical observation and the number of successful applications was small. The biggest hurdle to the exploitation of plant tissue culture was the absence of a well-defined understanding of how plant cells could be controlled to produce the target compounds. However, over the past few years, several important breakthroughs involving the use of biochemistry and molecular biology have once again raised the optimism that plant tissue culture will be able to produce important secondary metabolites in high yield.

In general, the results of many studies can be grouped into the following observations:

1. The cultured plant does not produce the same metabolites as the intact plant; sometimes compounds undetected in the intact plant are produced in the culture.

2. The yield of the desired metabolite is lower than that found in the whole plant.

3. Plant cells grow at a much slower rate than microorganisms.

The main approaches to research in this field vary from the empirical and descriptive, to the level of biochemical control and genetic improvement. These can be grouped into the following lines of interest:

1. Optimization of culture media; determination of the full range of metabolites produced and comparison with those of the intact plant.

2. Stability of production over age of tissue.

3. Elucidation of biosynthetic pathways.

4. Isolation and characterization of enzymes.

5. Biotransformation of exogenous compound.

6. Study of factors controlling expression of biosynthesis.

7. Scale-up of production of secondary metabolites.

8. Transgenic plants and clones.

As can be seen from this list, the disciplines and techniques involved in plant cell culture span the range from chemistry to biology to molecular biology.

Optimization of culture media and conditions

In general, there are two types of media used: one for growth and maintenance of the tissue, and another for secondary metabolite production. The determination of composition is empirical and unique to each plant. Components of media can be

divided into seven groups of constituents as listed in Table 1 (4).

Finetuning the individual variables and components is a very tedious approach. A comprehensively systematic variation of conditions is not ordinarily done and most researchers start from a number of standard formulations. Despite the numerous studies already done, there are still only a few general rules for the design of media and conditions for the production of secondary metabolites.

Table 1. Components of of culture media and other conditions which are important in plant tissue culture (4)

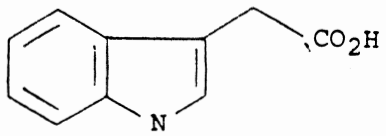
1. Major inorganic ions (N, K, Ca, Cl, Mg, P)
2. Trace inorganic ions (I, B, Mn, Zn, Mn, Mo, Cu, Co)
3. Fe source
4. Carbon source (sugar)
5. Organic supplements (vitamins)
6. Plant Growth Regulators (auxins, cytokinins, GA)
7. Miscellaneous (for example, precursors to metabolites)
8. Others: light (wavelength, duration), pH, temperature, choice of tissue and cell line, inducers.

Plant growth regulators (PGR) (Figure 3) influence cell division, growth, and differentiation, as well as secondary metabolite production. It has been suggested that PGRs are probably the single most important factor influencing production (4). In a number of cases studied, higher production of metabolites is observed in media of low concentrations of PGRs, although this may be at the expense of lower growth rates (5).

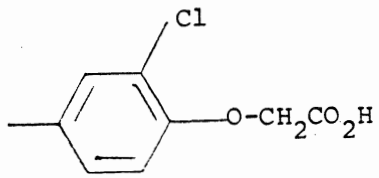
Identification of the full range of metabolites produced and comparison with those of the intact plant

One of the curious surprises of plant tissue culture is that very often the profile of metabolites produced *in vitro* as compared to the intact plant is significantly different. In some cases major metabolites of the intact plant are not produced in

Auxins: Promotes cell elongation

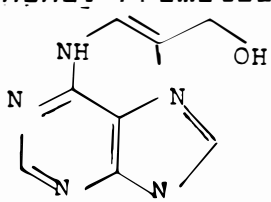


Indole-3-acetic acid

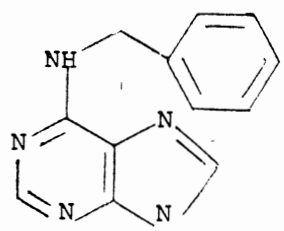


2,4-Dichlorophenoxy-acetic acid (2,4-D)

Cytokinins: Promotes cell division

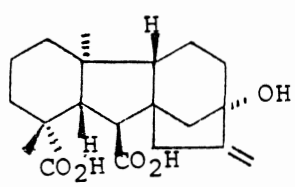


Zeatin



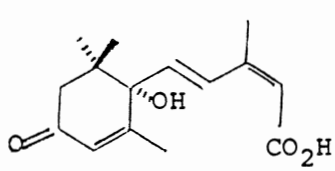
Benzyladenine (BA)

Gibberellic acids: Regulates cell elongation



GA_{53}

Abscisic acid (ABA): Promotes plant senesence



Ethylene: various effects

Figure 3. Some plant growth regulators and their effects on plant growth.

Table 2. List of some metabolites which have been produced from plant tissue culture.

a. Plant tissue cultures that do not produce the same metabolites as obtained from the intact plant.

Plant species	Metabolite	Use
Atropa belladonna	Atropine	brochial decongestant
Cinchona ledgeriana	Quinine	antimalarial
Digitalis lantana	Digoxin	cardiac drug
Papaver somniferum	Codeine	analgesic; antitussive
Rauwolfia serpentina	Reserpine	antihypertensive

b. High-yielding plant tissue cultures.

Plant species	Metabolite	Use
Coleus blumei	Rosmarinic acid	antihistamine
Coptis japonica	Berberine	antibacterial
Lithospermum erythrorhizon	Shikonin	dye, cosmetic

the cultures, and in other cases, compounds not previously observed in the intact plant are produced in culture. From a biological point of view, this should not be entirely surprising since the conditions existing in vitro as opposed those in the intact plant are very different. Table 2 summarizes some data on metabolite production comparing the intact plant with cell cultures. These observations suggest two important things:

1. In many cases, synthesis and accumulation of metabolites require some degree of morphological or biochemical differentiation not present in undifferentiated tissues. For example, special storage sites, such as vacuoles, may be necessary for bioaccumulation; certain membranes or organelles, such as the endoplasmic reticulum, may be necessary for the organization of loosely-associated enzyme complexes (5,6).

Evidence in support of this general hypothesis has built up over the past 25 years. There are a number of examples which show that there is a definite relationship between the development of the plant and the expression of secondary metabolites. For example, it is now suggested that alkaloid biosynthesis is strongly coordinated with the beginning of flowering; alkaloid production often slows down or ceases at this stage of development (7). The accumulation of some terpenes has been associated with vacuole development.

The implication for tissue culture is that, for secondary metabolite production, use of single cells may not be the best strategy, and that a certain amount of differentiation may be necessary.

2. The full genetic potential of the plant for secondary metabolite production is not being expressed completely, both in the intact plant and in culture. Under certain conditions, some of the metabolites are produced and others are suppressed. If this conclusion is correct, then the following questions arise: What determines metabolite expression? What are the control points for metabolite production? The tools for answering these questions lie in the fields of plant biochemistry and molecular biology.

Elucidation of biosynthetic pathways

Biosynthetic studies have attracted much interest not only for their scientific importance, but for their practical applications as well. Before the advent of the use of tissue culture, biosynthetic studies utilizing intact plants encountered many technical difficulties such as low incorporation of labeled compounds, presence of a large pool of metabolites which dilutes the label, channeling to other metabolic pathways, and others. Use of plant tissue culture avoids many of these limitations, and also provides easier access to the observation of enzyme function and enzyme purification.

The biosynthesis of a wide range of plant metabolites has been studied by tissue culture. In a number of cases, such as the monoterpenes and sesquiterpenes, the biosynthetic mechanism is known to the level of stereochemistry (8,9). Here, the elucidated pathway for flavonoid biosynthesis will be briefly outlined.

Flavonoids are a widely occurring family of metabolites, with well over several hundred different compounds all sharing the common C6-C3-C6 tricyclic ring system. The main sources of structural diversity arise from the varied substitutions possible, such as the oxidation level, O- and C-glycosidation, substitution

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of hydroxyl groups with various residues such as methyl, isopentyl, and acyl residues such as acetyl, malonyl, and aromatic acyl. The biosynthesis of flavonoids proceeds along a common pathway to the flavanone and then branches out to produce the various products. (Figure 4) (10).

The biosynthesis of flavonoids can be divided into two groups of enzyme systems which are closely linked in function and inducibility (11). Group I is the General Phenylpropanoid System and is comprised by three well-characterized enzymes. Figure 5 outlines the series of initial transformations from phenylalanine to the coumarines. Table 3 lists the Group I enzymes. Group II is the Flavonoid System with at least 13 enzymes already identified and studied to various levels of detail. The various products and enzymes of the Flavonoid System are outlined in Figure 6 and Table 4, respectively.

However, in addition to the study of biosynthesis, the problem of accumulation of metabolites also requires knowledge regarding the turnover and degradation of these compounds in the cell. It should be emphasized that plant compounds are not metabolically inert and may be present under steady-state conditions or because of stress. In addition, it should be realized that the definition of an "end product" is only a convenience and may be artificial one. There may be a continuous flow of material through various metabolic pools which are regulated according to the needs of the plant (12).

In the case of flavonoids, for example, it has been shown that breakdown is often mediated by plant peroxidases. For example, in *Mentha longifolia* eriodictyol-7-rutinoside is believed to be broken down into 5,7-dihydroxychromone-7-rutinoside. (13). It was also shown that kaempferol-3-O-beta-D-glucoside is readily broken down intracellularly, initially by oxidation of the 3-O-glucoside. Although the detailed pathway was not elucidated, labeling experiments indicate that the breakdown products were eventually incorporated into insoluble polymeric cell material. (Figure 7)

Isolation and characterization of enzymes

Since each metabolic step is mediated by a specific enzyme, the isolation and characterization of enzymes provides an essential key to understanding and controlling biosynthesis of secondary metabolites. Among the questions of interest are: What is the rate-limiting step? What are the inhibitors and modulators? How does the enzyme control/produce stereochemistry in metabolites? How are enzymes regulated? What is the specificity of

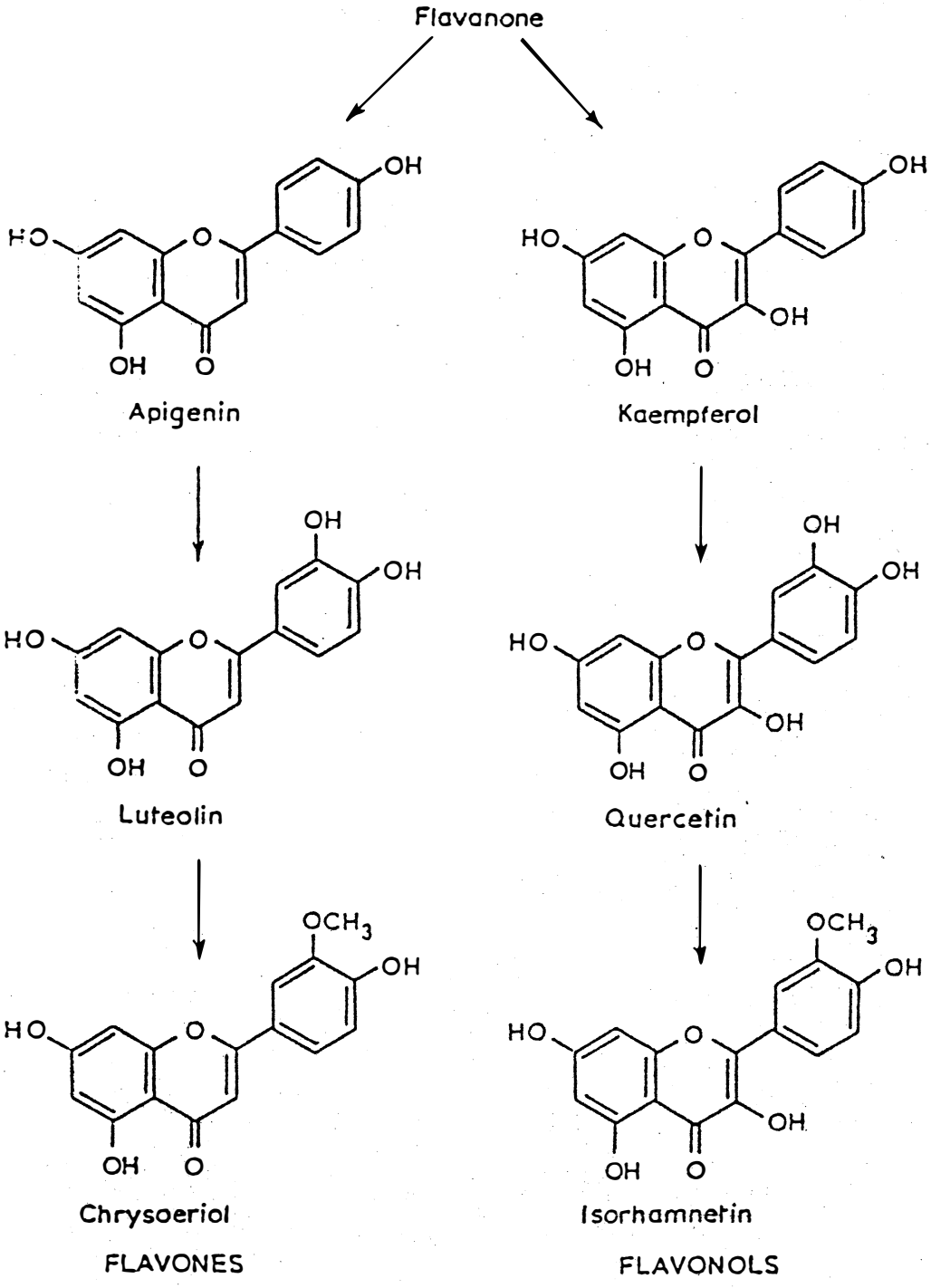


Figure 4. Family of flavonoids according to their biosynthetic relationships (from Ebel and Hahl brock. 1982).

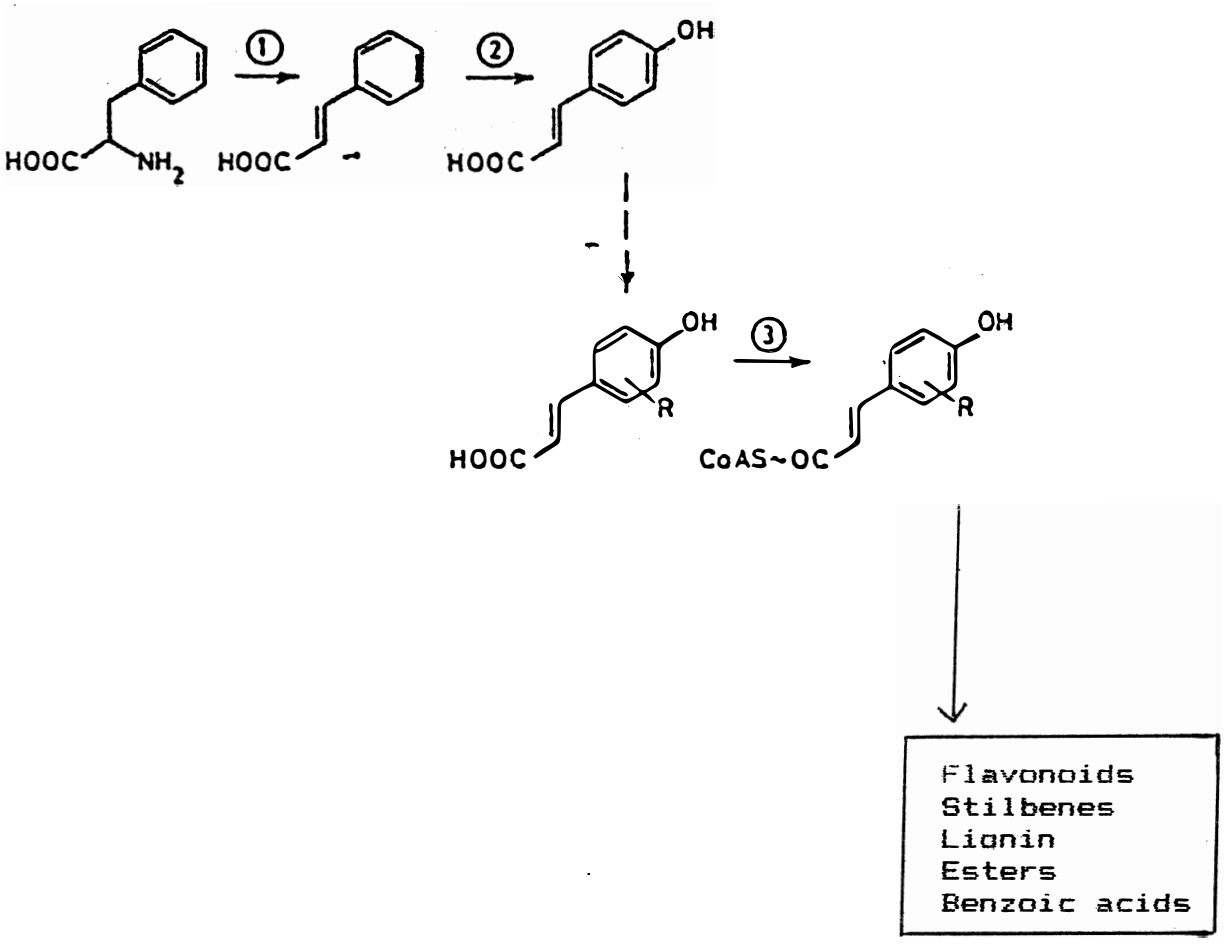


Figure 5. Group I: Transformations of the General Phenylpropanoid System. The corresponding enzymes are given in Table 3. (from Ebel and Hahlbrock, 1982)

Table 3. Enzymes of Group I: the General Phenylpropanoid System. (see Figure 5 for the corresponding transformation) (from reference 10).

Number	Name / E.C. number
1	Phenylalanine ammonia lyase (PAL), (4.3.1.5)
2	Cinnamate 4-hydroxylase (1.14.13.11)
3	4-Coumarate:CoA ligase (6.2.1.12)

Table 4. Enzymes of Group II: the Flavonoid System (see Figure 6 for the corresponding transformation) (from reference 10)

Number	Name / E.C. number
1	Acetyl-CoA carboxylase (6.4.1.2)
2	Chalcone synthase
3	Chalcone isomerase (5.5.1.6)
4	"Flavonoid 3-hydroxylase"
5	"Flavonoid oxidase"
6	"Flavonoid 3'-hydrolase"
7	S-Adenosyl-L-methionine:flavonoid 3'-O-methyl transferase (2.1.1.42)
8	UDP-Glucose:flavonoid 7-O-glucosyl transferase (2.4.1.81)
9	UDP-Glucose:flavonol 3-O-glucosyl transferase
10	UDP-Apiose/UDP-xylose synthase
11	UDP-Apiose:flavone 7-O-glucoside 2"-O-apiosyl transferase (2.4.2.25)
12	Malonyl-CoA:flavonoid 7-O-glucoside malonyl transferase
13	Malonyl-CoA:flavonol 3-O-glucoside malonyl transferase

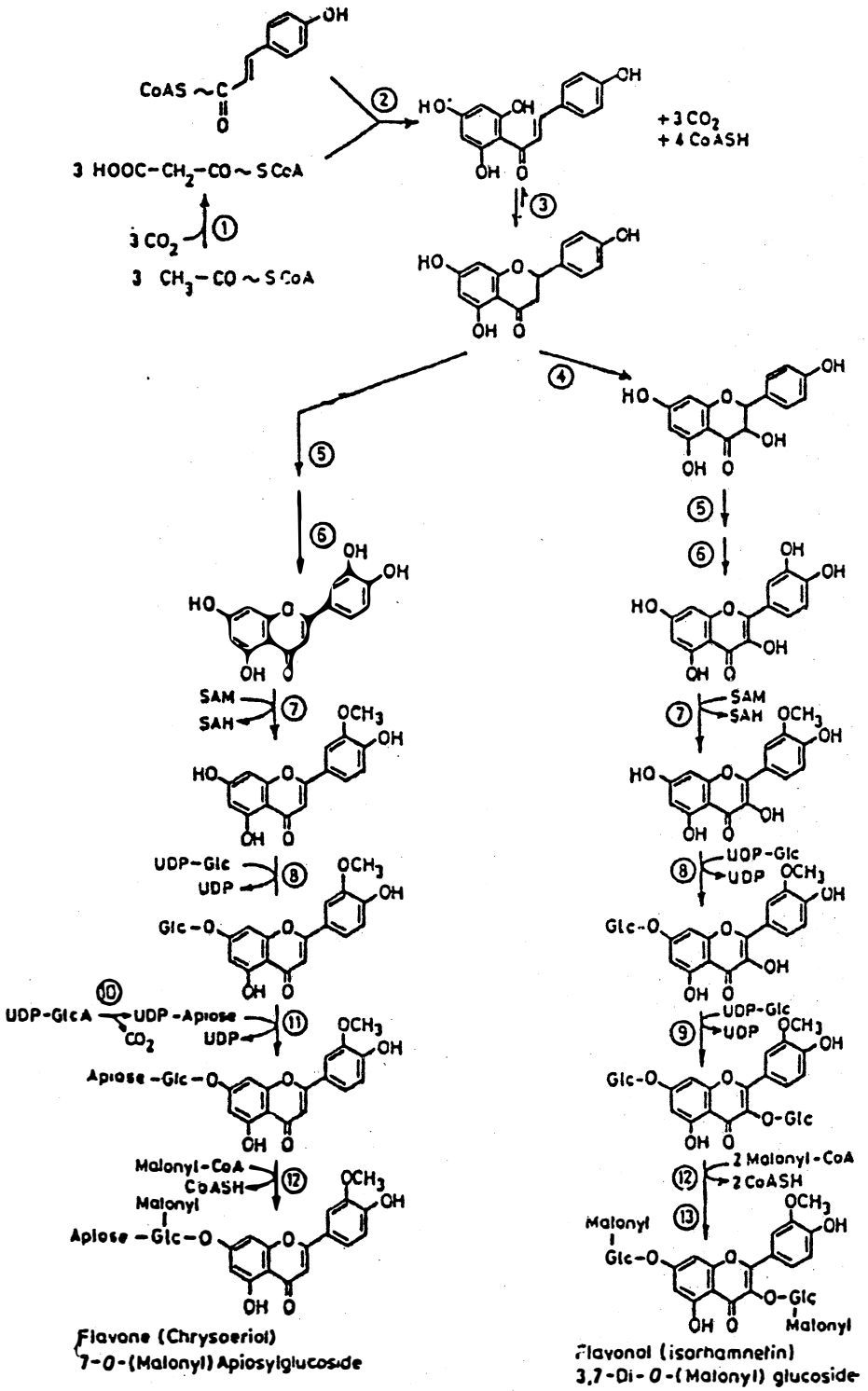
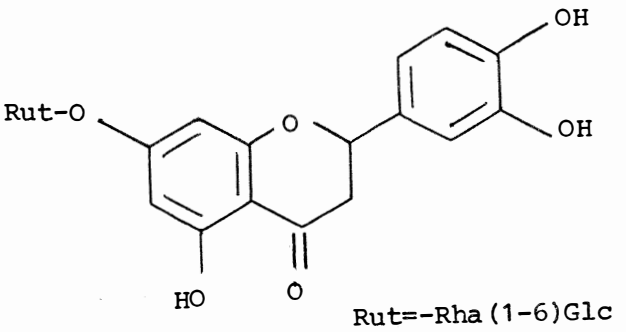
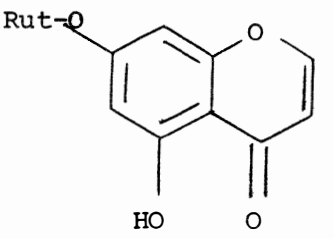


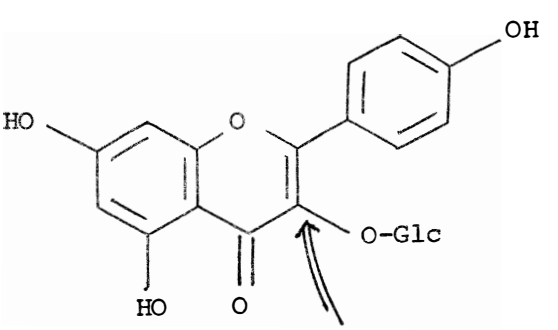
Figure 6. Group II: Transformations of the Flavonoid System. The corresponding enzyme are given in Table 4. (from Ebel and Hahlbrock, 1982)



Eriodictyol-7-rutinoside



5,7-Dihydroxychromone-7-rutinoside



Kaempferol-3-O-beta-D-glucoside

Figure 7. Breakdown of flavonoids in plants (13).

the enzyme? Various control mechanisms are possible: control at the enzyme level; control at the level of substrate supply; control at the level of **gene transcription:**

DNA ----> mRNA ----> enzyme

In the leaves of oat *(Avena sativa)* it appears that chalcone synthase is the rate-limiting enzyme for biosynthesis of flavonoids (14). However, in studies carried out with buckwheat, radish, and rye seedlings, it was concluded that the catalytic capacity of the enzyme (in this case, phenylammonia lyase, PAL) was not the determining factor, but it was the supply of substrate (phenylalanine) (15). A third possibility is that concentration of metabolite is regulated by the synthesis and breakdown of the enzymes themselves which is under the control of the gene.

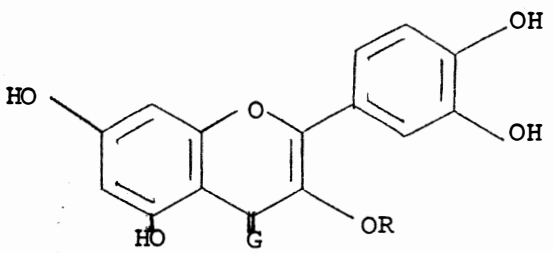
The purification of enzymes opens up two important avenues of of development: cell-free enzyme systems and a lead on further investigations on the plant's DNA. The former can be exploited for biotransformation applications; the latter can be used for investigations into the molecular biology of the plant

Biotransformation of exogenous compounds by plant tissue culture

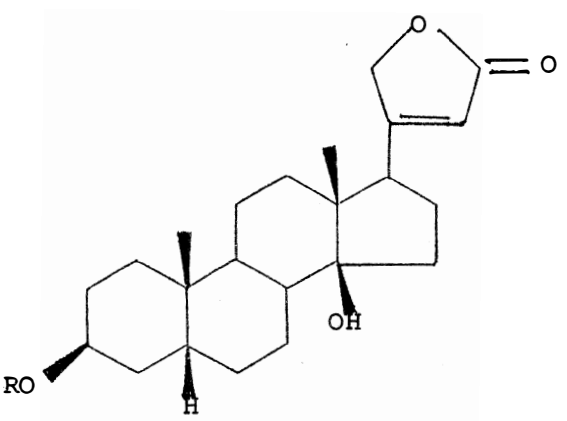
Plant enzymes are able to synthesize complex molecules with remarkable regio- and stereospecificity. Despite advances in synthetic chemistry, there remain many transformations for which plant enzymes are better. Biotransformation refers to the process wherein a compound is added either to a plant cell culture or to an isolated or immobilized enzyme in order to convert the compound to a specific product. In some cases, the added compound need not be contained in the parent plant. Among the chemical transformations that have been investigated are: glycosylation, oxidation-reduction of alcohols and ketones, hydrogenation of double bonds, and deacetylation (hydrolysis) (16).

Many plants possess a high capacity to glycosylate phenolic and steroidal substrates. Many reports have been made on the addition of glucose to specific sites using a number of flavonoids (16,17). Similar techniques have recently been applied to the glucosylation of cardenolide aglycones such as digitoxigenin. Glucose was added specifically to the 3-O position (Figure 8) (18).

Such technology is being exploited for the large-scale production of commercially valuable pharmaceuticals where difficult regio- or stereospecific steps can be carried out under



Quercetin -------> Quercetin-3-O-beta-D-glucoside
R=H R = beta-D-glucose



Digitoxigenin -----> Digitoxigenin-beta-D-glucoside
R=H R = beta-D-glucose

Figure 8 Biotransformation of exogenous substrates by plant tissue cultures. Examples of in-vitro glucosylation reactions (17,18).

mild conditions without the necessity of maintaining the cell. What is important to note is that, in many cases, the substrate itself need not be native to the plant. In fact, the substrate need not be a natural product. This simply extends the repertoire of available enzyme technologies.

Some factors controlling expression of biosynthesis .

A number of other factors have been shown to influence the expression of biosynthesis. It has been observed that various kinds of "stress" on cultured cells exert considerable influence on the secondary metabolism of the tissue. Examples of stress-induced regulation are induction, catabolite repression, and feed-back inhibition. Here, two such factors will be discussed: light and elicitors. In both cases, it has been shown that these influences are made at the level of transcription of mRNA.

Light plays an important role in the induction of certain enzymes. In a series of studies on the biosynthesis of flavonoids in parsley, it was shown that light activates mRNA transcription for the de novo synthesis of phenylammonia lyase (PAL), chalcone synthase and flavone synthase, three of the six enzymes required for the biosynthesis of flavonoids (19). However, in a recent report on the enzymatic regulation of shikonin biosynthesis in *Lithospermum erythrorhizon* cell cultures, it was shown that PAL, which is known in other plants to be a light-inducible, was not influenced by light (20).

Of current interest is the role of specific compounds, called **elicitors**, in the activation of the biosynthesis of chemical defense substances. These elicitors can be compounds from an attacking organism (for example, fungus, bacteria, or insect) or they can be fragments from the plant's own cell wall which signals to the plant that its cell wall defense has been damaged (21). It is then reasonable to assume that by nature's design, the compounds induced by elicitation must have some biological activity against the attacking organism; such plant metabolites are called **phytoalexins.**

A number of phytoalexins, such as isoflavones (22) and coumarins (23) have been shown to be produced by fungal elicitation. In a wider context, understanding the chemical ecology of the plant can give important hints regarding the elicitation of specific compounds in plants. (24)

The discovery of elicitation has been particularly useful in several ways: it promotes the biosynthesis of specific compounds

which are likely to have some biological activity, and it gives researchers the opportunity to study the whole process of biosynthesis starting from gene expression to product formation. For example having identified the phytoalexins, the next steps are to trace the biosynthetic pathway, isolate the enzymes produced, and elucidation of the cDNA (complementary DNA) which encodes for these enzymes. This enables entry into **recombinant DNA** research.

Scale-up of production

The specific problems faced in the large-scale production of secondary metabolites from plant cells as compared with microbial or yeast fermentation arise mainly from the greater complexity of plant cells and slower growth. For viable commercial production, the target chemicals have to be of high value. Economic studies estimate that at an annual production of 10,000 kg/year, the break-even point can be reached if the selling price is US$1368/kg (20).

Compared to microbial fermentation, plant cell fermentation has had a lower success rate. Mitsui Petrochemical Industries scored the first notable commercial success in its development of the production of shikonin using *Lithospermum erythrorhizon cells* in suspension culture. Shikonin is used mainly in Japan for the treatment of burns, as a silk dye, and cosmetic. It is extracted from the roots of *L. erythrorhizon* and harvesting can be done only after about 4 years. In the Mitsui process, a single production run of 14 days in a 750-liter fermentor is able to yield as much shikonin as a 18 hectare field after 4 years (20).

At present much work is being carried out to develop large-scale production of other valuable, and difficult-to-synthesize, plant secondary metabolites such as alkaloids and cardiac glycosides. This technology poses a direct challenge to the traditional approach of agricultural production of these plantmetabolites.

Transgenic plants

Gene technology has been applied in plants to enhance the production of secondary metabolites. Research in this field generally follows the strategy of: a. elucidation of biosynthetic mechanism; b. isolation and characterization of important enzymes of biosynthesis; c. determination of the induction of gene expression; d. identification, isolation, and cloning of the corresponding cDNA; e. gene synthesis and/or genetic transformation by insertion of foreign genes (25,26). Such studies have been also able to identify the importance of differentiation

or development of certain tissues in the accumulation of metabolites.

Research has been carried out on the transfer and expression of foreign genes in *Digitalis purpurea* (foxglove) and *Glycyrrhiza uralensis* (licorice), two plants of considerable pharmaceutical importance. This technology opens up new possibilities for the improvement of yields of secondary metabolites by genetic manipulation.

Recently, species of a soil pathogen, *Agrobacterium rhizogenes* and *A. tumifaciens*, were shown to have the ability to transfer and integrate its genomic information into the plant DNA forming "hairy roots" or crown galls. (21,26-30) This process of gene transfer has been extensively used as vectors to transfer foreign genes into plants. It has been applied to the production of tannins (29), terpenes (28,30,31), and alkaloids (27,30,31).

Conclusion

The field of plant tissue culture for the production of secondary metabolites spans the range of the empirical, trial-and-error approach, to the use of biochemistry, enzyme chemistry, plant physiology, biotechnology, and molecular biology. While a detailed understanding of the control of biosynthesis has remained elusive, significant progress can be expected in the near future. The difficulties can be attributed to the greater complexity of plants and to the lack of knowledge regarding the specific role that these metabolites play in plants.

With the explosive progress in biochemistry, biotechnology and molecular biology, it may just be a matter of time before many of the hurdles to the biosynthesis of plant secondary metabolites can be overcome. It is not unreasonable to expect that this technique will become the industry standard for the production of pure secondary metabolites paralleling the way that antibiotics are being produced by fermentation.

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