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J. Nat. Prod., **1993**, 56 (2), 186-207• DOI: 10.1021/np50092a003 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 2, pp. 186-207, February 1993

PLANT CELL BIOTECHNOLOGY FOR THE PRODUCTION OF ALKALOIDS: PRESENT STATUS AND PROSPECTS[1]

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ABSTRACT.—The culture of plant cells on a large scale in bioreactors has been shown to be feasible. The price of a plant cell biotechnological product is mainly governed by the slow growth of plant cell cultures, making the depreciation costs of the bioreactor the major cost-determining factor. A review of the production of the economically important alkaloids in plant cell cultures shows that presently only berberine and sanguinarine are being produced.

Important factors to be considered in connection with accumulation of alkaloids are biosynthetic rate, accumulation site, and catabolism. Recent studies in the field of the regulation of the biosynthesis of terpenoid indole alkaloids on the level of genes and enzymes are reviewed, showing that it is feasible to clone genes from secondary metabolism and express these in various other plants. Concerning storage, it seems that compartmentation also plays a role in the regulation of alkaloid biosynthesis. Furthermore, catabolism of terpenoid indole alkaloids in cell cultures is an important factor, at some point even equalling the rate of de novo biosynthesis.

The ongoing studies on regulation of alkaloid biosynthesis might eventually lead to transgenic plants or plant cell cultures with an improved productivity of the desired compounds. This knowledge is also of interest in connection with studies on the role of secondary metabolism for plants, and may contribute to a better understanding of resistance of plants to diseases and various herbivores.

Of all known natural products, about 20% (i.e., about 16,000) are classified as alkaloids. For many of these alkaloids biological activities have been reported, but presently only about 30 are commercialized (1). Most of these are medicines, but some are used as flavoring, poison, and model compounds for pharmacological studies. These alkaloids can be qualified as specialty chemicals, as their worldwide production volume is limited; alkaloids such as quinine and quinidine have a yearly production of 300–500 metric tons, ajmalicine about 3600 kg, and compounds like vinblastine and vincristine in the kilogram range only (2,3). The amounts of plant material needed for the extraction of these compounds are, compared to agricultural crops, very small. For the examples mentioned this is in the order of 5000–10,000 metric tons of *Cinchona* bark for the extraction of quinine and quinidine and 200–300 tons of *Catharanthus roseus* roots for the production of ajmalicine. The value of each of the markets of the major alkaloids can be estimated to be in the range of several hundred million dollars (4).

These specialty chemicals are now produced by extraction from plant material that is cultivated or sometimes even still collected in the wild. There are several problems connected with this production method. Variable quantities and qualities of the plant material, plants that need to grow several years before they are ready for harvesting (e.g., *Cinchona* bark), and over-collecting of endangered species (e.g., *Taxus brevifolia*)

[1]Presented as a plenary lecture at the "Applications of Biotechnology" Symposium of the 33rd Annual Meeting of the American Society of Pharmacognosy, Williamsburg, Virginia, July 26–31, 1992.

are just a few of the problems connected with the production of these specialty chemicals. For such high-value products alternative production methods are thus of great interest. As a consequence a lot of work has been done on the synthesis of natural products as an alternative to extraction. Although complete synthesis for most natural products has been shown to be possible, it is usually economically not feasible. Semisynthesis, starting with more readily available precursors, has been successful in some cases, e.g., the coupling of monomers for the production of the dimeric alkaloid vinblastine (5). Also novel useful compounds may be developed in this way, e.g., taxotere as a result of the work on the synthesis of taxol (6).

Due to their complex structures, alkaloids are still most efficiently produced by the plant. Therefore, in the past two decades one has focused on plant cell biotechnology as a possible alternative production method, using cultured cells rather than plants. Here a review will be given of the present status of plant cell biotechnology for the production of alkaloids. Furthermore, the strategies presently followed to improve production to commercially interesting levels will be described using some of our own studies as illustrations.

For industrial production two questions are crucial: Can plant cells be grown in large fermenters? Is the price of a product from such a large-scale plant cell culture competitive with existing production methods?

Here we will first address these general points before making an analysis of the situation for the industrially important alkaloids.

LARGE SCALE PRODUCTION.—Scaling up has been studied since the first successful in vitro growth of plant cells and tissues was described almost 50 years ago. The first patents were taken by Routien and Nickell in 1956 (7). The first successes were published in 1960 by Tulecke and Nickell (8), culturing cells of various plant species in a 134-liter bioreactor. In 1977 Noguchi *et al.* (9) reported the culture of tobacco cells in a 20 m^3 tank, mixed by aeration.

In the past decade most work on large-scale culture concerned the use of various types of low-shear bioreactors (e.g., airlift bioreactors), because plant cells were thought to be very sensitive to shear forces occurring in stirred-tank-type bioreactors. In doing so, little attention was paid to the cause of the supposed shear-sensitivity of plant cells. In fact, recent studies by Scragg and co-workers (10,11) and Meijer and co-workers (12,13) showed that shear-sensitivity of plant cells is not a general problem. On the contrary, many plant cell cultures are shear-tolerant and can be grown without any problem in stirred tanks. This finding is supported by examples from practice. The industrial production of shikonin is performed in 750-liter stirred-tank bioreactors (14), and in Germany the Diversa Company has successfully grown several types of plant cell cultures in 60 m^3 working-volume stirred tanks (15). For the industrial applicability of plant cell cultures this is of great importance, as in the fermentation industry stirred tanks are presently being used almost exclusively. Large investments in all sorts of new ingenious bioreactors, as reported for plant cell cultures in the past years, would be a major constraint for the commercialization of plant cell biotechnology.

Why some cell lines are shear-sensitive and others shear-tolerant is not yet known, although Meijer and co-workers (12,13) noted that "healthy" good-growing cell cultures were more shear-resistant than apparently "stressed" (e.g., rapidly browning) cell cultures.

The next question to address is the economic feasibility. Several cost price calculations have been published (16–20). In Figure 1 the calculations made by Goldstein have been combined with the actual cost price of a series of compounds (3,20). This figure

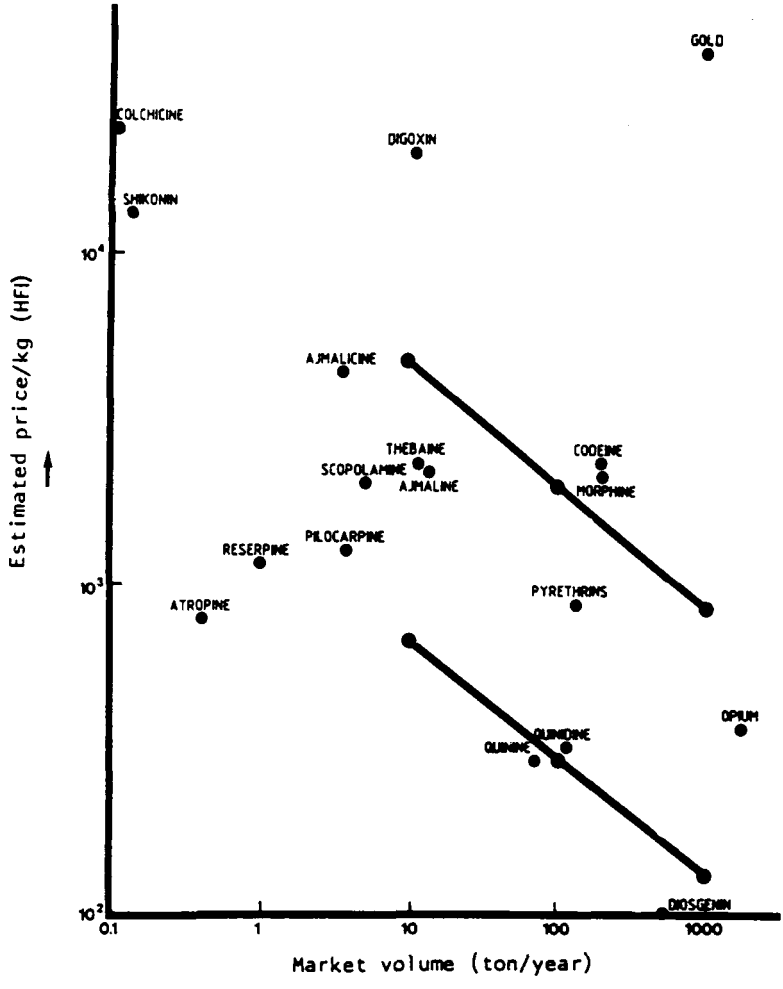
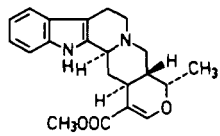


FIGURE 1. Price and market volumes of some natural products and production costs by plant cell biotechnological production. The upper line represents present day technology, the lower line represents a tenfold improvement in productivity (16,20).

shows the estimated market volumes of a series of compounds and their estimated price per kg. The two lines depict the costs of a plant biotechnological production as calculated by Goldstein *et al.* (16). The upper line represents the production for a natural product with current technology, the lower line the costs if a tenfold improvement of the productivity were achieved. From both lines it is evident that for only a few products would the market price warrant a plant biotechnological production. Calculations for a specific product have been made as well. Fowler and Stepan-Sarkissian (17) calculated that ajmalicine [**1**] produced by cell cultures of *Catharanthus roseus* would cost



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$35,000/kg for the production of 375 kg/year. Drapeau *et al.* (18) estimated a price of $3,215/kg for the same alkaloid. Van Gulik and co-workers (19,20) made a detailed feasibility study of a plant cell biotechnological production, using ajmalicine as a model. Two processes were compared: (a) batchwise growth and production and (b) continuous extraction of alkaloids from a cell culture releasing the product. In Table 1

TABLE 1. Parameters Used in Cost Price Calculation Ajmalicine [1] Production by Cell Cultures of *Catharanthus roseus* (20).

Design basis	
Yearly production	3000 kg
Product loss during isolation	20%
Effective production days/year	300
Growth parameters	
Specific growth rate	$0.029\ h^{-1}$
Initial biomass (dry wt)	$2.5\ kg/m^3$
Inoculation ratio	1:7
Dry wt yield from glucose	0.61 kg/kg
Maintenance energy requirement	0.0066 kg/kg per h
Maximum oxygen uptake rate	$0.0154\ kmol/m^3$ per h
Single use of biomass	
Final biomass concentration (dry wt)	$40\ kg/m^3$
Final biomass concentration (fresh wt)	$320\ kg/m^3$
Final ajmalicine content after 21 days production period	0.009 kg/kg
Repeated use of biomass	
Specific productivity, spontaneous product release	2.36×10^{-5} kg/kg per h
Ajmalicine concentration in biomass in case of forced release	0.009 kg/kg

the parameters used for these calculations are summarized, and in Table 2 the cost prices are calculated. If the same calculations are made for berberine [2], an alkaloid that is produced at much higher levels (up to 7 g/liter) (14, 21–23), the price is considerably lower (Table 3). All these prices do not include downstream processing and labor. The most important conclusion is that a simple batchwise culture is the most economical. Moreover, most of the costs are in the depreciation of the equipment, due to the slow growth and low product formation rates of plant cells. It once more shows that the already existing stirred bioreactors are the most applicable for the large scale production, as they do not require new investments.

In conclusion, plant cells can be cultured on a large scale, and although the price of the products obtained will be high, this price is still in the order of magnitude of that of many specialty chemicals.

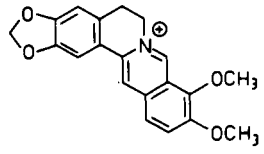
OPTIMIZING PRODUCTIVITY.—In order to achieve an industrial production one has to obtain a stable, high-producing cell line of the plant of interest. For this two approaches are being used: (a) screening and selection for high producing cell lines and (b) optimization of growth and production medium.

In a screening program a large number of cell lines are set up, derived preferably from high-producing plants. Subsequently the production of the compound of interest by these cell lines is determined, and the best producing cell lines are selected. High

TABLE 2. Breakdown of Costs of Different Processes for Large-scale Production of Ajmalicine [1] by Cell Cultures of *Catharanthus roseus*.[a]

	Product extraction from cells	10× improved	Product release	Permeabilization (DMSO)
Production reactor volume (m^3)	6×145	6×15	6×250	6×335
Biomass reactor (m^3)	37	3.8	82	10
Storage tanks (m^3)	3×10	3×1.2	7×150	7×150
Depreciation costs in $/year	3×10^6	1.1×10^6	5.4×10^6	5.4×10^6
Medium costs in $/year	0.75×10^6	0.1×10^6	3.3×10^6	5.1×10^6
Energy costs in $/year	0.75×10^6	0.08×10^6	1.5×10^6	1.5×10^6
Total costs in $/year	4.5×10^6	1.3×10^6	10.2×10^6	12×10^6
Costs $/kg	1500	430	3400	4000

[a]Comparing a cell culture in which the alkaloid is to be extracted from the biomass, the same process but with ten times more productive cells, or from the medium after spontaneous or forced release (20).



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production in plants does not necessarily correlate with high production in cell cultures, as was shown for berberine-producing *Coptis japonica* cell cultures for which in fact the highest producing cell line was obtained from a low-producing plant (21,22). A further screening can be done at the level of cell aggregates or even single cells or protoplasts. In case of the *Co. japonica* cell cultures it was found that at least three to four cloning steps were necessary before a stable, high-producing strain was obtained (23). For other plants, such as *Catharanthus roseus*, selection of high-producing strains was possible, but this trait was not stable (24). Stability is a major problem encountered not only in plant cell cultures but also in other biotechnological processes such as antibiotic production by means of microorganisms.

For selection, conditions are created in which only a certain desired cell type will survive. For example cells of *Ca. roseus* have been grown on media containing the toxic compound 4-methyltryptophan, to obtain cell lines that have a higher alkaloid production capacity. Only cells that contained a high level of tryptophan decarboxylase, and thereby were able to convert the selection agent into the less toxic 4-methyltryptamine, survived. The selected cell lines did have a higher tryptamine production, but only in one single case was terpenoid indole alkaloid production increased too (25).

The other important approach to increasing production is to improve the growth and production conditions; i.e., various media compositions are tested to increase biomass density and product levels in the culture. In many cases production does not parallel growth. Hence, a two-step system is often used. First the biomass is grown under optimal conditions, then the medium is changed into a production-inducing

TABLE 3. Cost Calculated for the Production of 3000 kg/year of Berberine [2] by Means of Plant Cell Cultures.

Single use of biomass (*Coptis japonica*, 3.5 g/liter)	360 $/kg
Spontaneous release of product, discontinuous process (*Thalictrum minus*, 1.4 g/liter)	760 $/kg
Spontaneous release of product, continuous process (*Thalictrum minus*, 1.4 g/liter)	850 $/kg
Immobilized cells (*Thalictrum minus*)	600 $/kg

medium. Such media have been reported by Zenk *et al.* (26) and Knobloch *et al.* (27) for the induction of ajmalicine production in *Ca. roseus*.

Light is a factor that may increase production for certain cell lines. However, in large-scale production in stirred tank bioreactors such conditions are difficult and costly to realize. Therefore, production conditions on small scale should preferably be optimized with dark-grown cultures. Similarly, in transferring results from batchwise cultures in shake flasks to bioreactors it has to be kept in mind that the gas phases in the two systems are completely different, thus possibly influencing the productivity. This may be illustrated by the recent finding that productivity of *Ca. roseus* cell cultures in bioreactors is drastically improved by recirculation of the air used for the aeration of the cells in the bioreactor (28).

For certain plants it has been shown that the use of differentiated cultures does result in considerably higher production of the compounds of interest (see below). Such cultures (e.g., root or shoot cultures) are obtained by applying certain combinations of plant growth hormones. In this context the so-called hairy root cultures are worth mentioning. These cultures are obtained by infection of the plant tissue with *Agrobacterium rhizogenes*, a soil bacterium that introduces root-inducing genes into the plant cells. These genes transform the cells into hormone-independent tumorous roots that have production of secondary metabolites similar to that of the plant roots. The growth rate is for some species comparable with that of the plant cell cultures (see below). A major problem associated with organized cultures remains their growth on a large scale. Wilson *et al.* (29) and Rodriguez-Mendiola *et al.* (30) have proposed different solutions for the large-scale culture of hairy roots.

Another option for increasing secondary metabolite production that has been successful in several cases is elicitation. By adding elicitors such as cell wall constituents of microorganisms, enzymes (e.g., cellulase, pectinase), or heavy metals, certain biosynthetic pathways are induced (e.g., sanguinarine, see below). Probably the compounds formed upon elicitation do play a role in plant defense mechanisms, e.g., as phytoalexins. Recently Gundlach *et al.* (31) showed that jasmonic acid, functioning as a signal molecule in the transduction chain of the elicitation response, can be used to induce these pathways as well. Among others the production of sanguinarine in *Eschscholtzia californica* and of raucaffricine in *Rauvolfia canescens* could be induced.

PRODUCTION OF ALKALOIDS.—Having shown the technological feasibility, the next question is whether alkaloids are, after optimization of the cell cultures as discussed above, produced in sufficiently high levels by cell cultures to enable a commercial production. In Table 4 the production of the most important alkaloids is summarized. In the following paragraphs the situation for some alkaloids will be briefly described.

Nicotine.—From a commercial point of view nicotine is probably a compound of lit-

tle interest, but tobacco has long been a model plant for plant cell and tissue culture studies. Production of alkaloids has been studied in various types of tobacco cell cultures. Despite all the efforts, so far no stable high-nicotine-producing cell line has been developed. Reported alkaloid productions range from 0.005% to 2.9% of dry wt. (32) in cell suspension cultures. Root and particularly hairy root cultures of various tobacco species have been reported to produce considerable amounts of alkaloid (20,33). Tobacco cell suspension cultures have been grown on a large scale (20 m^3) (9); hairy root cultures have not yet been grown on a large scale.

Tropane alkaloids.—For many years unsuccessful attempts have been made to pro-

TABLE 4. Prices[a], Plant Source, and Product Yields by Means of Alternative Biotechnological Methods of Some Commonly Used Alkaloids.[b]

Alkaloid	Plant source	Price US$/gram	Production by cell cultures[c]	
			Source	Yield
Ajmalicine [1]	*Catharanthus roseus*	37.00	*Ca. roseus* SC	0.2 g/liter
Vinblastine	*Ca. roseus*	10,530.00	*Ca. roseus* ShC	traces
Vincristine	*Ca. roseus*	25,200.00	*Ca. roseus* ShC	traces
Ajmaline	*Rauvolfia* sp.	10.00	*Rauvolfia* SC	0.04 g/liter
Reserpine	*Rauvolfia* sp.	8.30	*Rauvolfia* SC	0.002 g/liter
Rescinnamine	*Rauvolfia* sp.	18.30	no data available	
Vincamine	*Vinca* sp.	19.70	*Vinca minor* SC	3.3 g/liter
Quinine	*Cinchona* sp.	0.50	*Cinchona* SC	trace
Quinidine	*Cinchona* sp.	0.90	*Cinchona* SC	trace
Ellipticine	*Ochrosia elliptica*	2630.00	*O. elliptica* SC	0.005% dry wt
Camptothecine	*Camptotheca acuminata*	480.00	*Cam. acuminata* SC	0.00025% dry wt
Emetine	*Cephaelis ipecacuanha*	26.30	*Cephaelis* RC	0.3–0.5% dry wt
Physostigmine	*Physostigma venenosum*	52.60	No reports on pctc.	
Pilocarpine	*Pilocarpus microphyllus*	17.00	No reports on pctc.	
Caffeine	*Coffea, Thea*	0.08	*Coffea* SC	0.48 g/liter
Theobromine	*Theobroma*	0.60	*Coffea* SC	
Atropine	*Atropa belladonna*	3.50	*Atropa* HR	0.1–0.2 g/liter
	Datura sp.		*Datura* HR	0.1–0.2 g/liter
	Hyoscyamus sp.			
Scopolamine	*Duboisia* sp.	17.30	*Duboisia* HR	0.08 g/liter
	Hyoscyamus sp.		*Hyoscyamus* HR	0.4% dry wt
Berberine	*Coptis japonica*	11.00	*Cop. japonica* SC	7 g/liter
	Berberis		*Thalictrum* SC	0.87 g/liter
Sanguinarine [3]	*Eschscholtzia*	48.00	*Eschscholtzia* SC	0.16 g/liter
	Papaver somniferum		*Papaver* SC	0.25 g/liter
Tubocurarine	*Chondrodendron tomentosum*	84.00	No reports on pctc.	
Morphine	*Papaver somniferum*	340.00	*Pa. somniferum* SC	0.25 g/liter
Codeine	*Pa. somniferum*	17.00	*Pa. somniferum* SC	0.25 g/liter
Nicotine	*Nicotiana* sp.	1.00	*Nicotiana* SC	0.36 g/liter
Colchicine	*Colchicum autumnale*	50.00	*Col. autumnale* CC	0.0006%

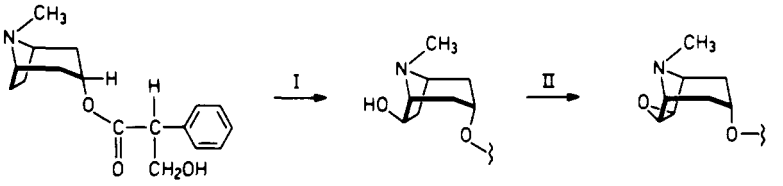
[a]Prices are based on 1990 catalogues of major suppliers of specialty chemicals.

[b]Results as reported in literature, as reviewed above, and in Verpoorte *et al.* (20).

[c]Abbreviations: pctc, plant cell and tissue culture; CC, callus culture; SC, cell suspension cultures; RC, root cultures; HR, hairy root cultures; ShC, shoot cultures.

duce tropane alkaloids by means of cell suspension cultures (20). Only root cultures were found to be able to produce these alkaloids. Transformation of tropane-alkaloid-producing plants with *Agrobacterium rhizogenes* readily yields the so-called hairy roots, which can easily be cultured in vitro and produce similar alkaloid levels as the plant. *Atropa belladonna* hairy roots produce alkaloids up to levels of 1.3% of the biomass dry wt (34). In hairy roots of *Datura* species the alkaloid contents varied from 0.2 to 0.6% (dry wt) (35); in hairy root cultures of various *Hyoscyamus* species the alkaloid content ranged from 0.1 to 0.7% (dry wt) (35). Root cultures of *Hyoscyamus* species produced alkaloid at levels of 0.2–0.4% (dry wt) (36). It is still a long way to a commercial production of tropane alkaloids, as the levels in the cultures are not higher than in the parent plant. Although some experiments on a 30-liter scale culture of hairy roots have been reported (34), no experience exists with larger scale production.

Extensive studies are being made on the enzymes and the encoding genes responsible for the conversion of (−)-hyoscyamine into the more valuable scopolamine (Figure 2). The gene encoding for hyoscyamine 6β-hydroxylase, catalyzing the first step in this conversion, has been cloned (37,38). Eventually this achievement may result in plants or cell cultures with an improved production of scopolamine.



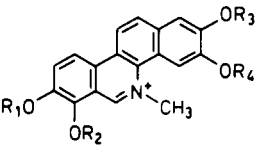
(−)-Hyoscyamine 6β-Hydroxyhyoscyamine Scopolamine

I hyoscyamine 6β-hydroxylase
II 6β-hydroxyhyoscyamine epoxidase

FIGURE 2. Bioconversion of (−)-hyoscyamine into scopolamine.

Opium alkaloids.—Because of their economic interest, opium alkaloid production by means of plant cell cultures has been extensively studied (20). Particularly the production of the morphinan alkaloids codeine and morphine in a closed system would be of great interest in connection with the legal control of the production of morphine. So far, however, no success has been achieved. Only in two cases have high levels of alkaloids been claimed. All other studies report very low levels in the various types of cultures (callus, suspension, root, and shoot). Production of morphinan alkaloids occurred only in the case of cytodifferentiation leading to laticifer type of cells (39).

Sanguinarine [3].—Cell cultures of *Papaver somniferum* and *Papaver bracteatum* do not produce any morphinan alkaloids, but they are capable of producing considerable amounts of benzophenanthridine alkaloids, with sanguinarine [3] as the major component (40). Because of commercial interest for this compound, its production was studied in more detail. The finding that addition of elicitors such as fungal cell wall constituents induced sanguinarine biosynthesis (41) was a major breakthrough in the further development of an industrial process. About 50% of the alkaloid was excreted into the medium. Based on this finding, a process was designed in which immobilized cells of *P. somniferum* are exposed to a fungal elicitor for 72 h, after which the medium is changed, and the cells are allowed to recover before they are again elicited. San-



$R_1+R_2=R_3+R_4=-CH_2-$

3

guinarine can then be extracted from the medium. Several of such cycles were possible with the same batch of cells (42–45). The total production obtained was 375 mg/liter in three cycles of 21 days. The production per unit of time is thus rather low when compared with berberine (see below).

Sanguinarine and related alkaloids can also be found in cell cultures of *Eschscholtzia californica* (20,46) and *Macleaya* species (20,47,48).

Berberine.—This alkaloid is widely used in Asia as a drug, usually in the form of crude plant extracts. Berberine has been found in cell cultures of a number of plants (20). Highest production levels were observed in *Coptis japonica*. By an extensive screening program and optimization of the culture conditions yields of 7 g/liter have been achieved, the highest yield for a product from plant cell cultures ever reported. These levels were reached in a stirred bioreactor in which the culture reached a very high biomass density (75 g/liter dry wt) containing 10% of alkaloid (14, 21–23, 49). As berberine is present in a mixture with others, thereby hampering the isolation, further studies were made on other plant species for the production of berberine. *Thalictrum* species proved to be of interest in this respect, especially *Thalictrum minus* as cell cultures of this plant produced berberine and excreted most of the alkaloid into the medium. By means of screening cell lines, a strain with a 350 times increased alkaloid production could be obtained (50). Yields of 400–800 mg/liter could be achieved. Growth and optimum alkaloid production could not be achieved in the same medium (51,52). Calcium alginate immobilized cells of *Th. minus* were shown to be suitable for a semicontinuous production; yields of 50 mg/liter/per day could be obtained during a continuous operation of 60 days (53,54). *Thalictrum rugosum* was also found to produce considerable amounts of berberine, particularly after elicitation with a yeast-derived elicitor preparation (55–57).

Cinchona *alkaloids.*—The production of alkaloids in *Cinchona* cell cultures is very low (20,58). With an increased level of differentiation, alkaloid levels increase as well (59). Some root and shoot cultures have been reported that produce 0.1–1 mg alkaloid per gram dry wt. Compact globular structures, which are cell aggregates with a diameter of about 0.5–1 cm, have similar production levels (59). Although hairy root cultures of *Cinchona ledgeriana* have been reported, these cultures are not promising as they showed very slow growth. Moreover, transformation with *Agrobacterium* of cells of *Cinchona* species occurs with very low incidence. In general, *Cinchona* species can be considered as recalcitrant with regard to plant cell and tissue culture.

Indole alkaloids.—Most of the plant cell biotechnology work in the field of terpenoid indole alkaloids concerns *Ca. roseus* and *Rauvolfia* species. In fact, *Ca. roseus* has developed into a major model system for plant cell biotechnological studies. Although much effort was put into the production of the dimeric alkaloids vinblastine and vincristine by means of *Ca. roseus* cell cultures, this goal has not been achieved. Only in shoot cultures (60,61) and occasionally in callus cultures (61) have these alkaloids been detected. One of the monomeric alkaloids from which the dimers are biosynthesized,

catharanthine, is produced in considerable amounts in cell suspension cultures. A production of 230 mg/liter after 1 week of growth, using a high inoculum density of 5-methyltryptophan-resistant cells, was reported (62). Vindoline, the alkaloid that provides the other part of the dimeric alkaloids, is produced only in differentiated tissues (63,64). Low levels of vinblastine have been found in hairy root cultures of *Ca. roseus* (65). With crude enzymes isolated from *Ca. roseus* cell cultures, it has been possible to produce anhydrovinblastine from catharanthine and vindoline (66,67). Even better yields could be obtained by using peroxidases (68,69). Recently these studies have resulted in a biomimetic two-step synthesis of vinblastine from the two precursors mentioned (62).

Although *Ca. roseus* cell cultures produced no dimeric alkaloids, they do produce ajmalicine and serpentine. In fact most of the work on the cell cultures of this plant concerns the production of these alkaloids, which are used in the treatment of hypertension. By screening and selection, high producing cell lines have been obtained (e.g., 20, 26, 70, 71). The highest level obtained was 264 mg/liter of ajmalicine together with 77 mg/liter of its oxidation product serpentine (26). These results were obtained by inoculation on an induction medium to increase the alkaloid production. High productivity was found to be an unstable trait (24). Recent reviews (20,72,73) have discussed the various factors influencing alkaloid production.

Several authors have studied the culture of *Ca. roseus* cells in bioreactors. One important conclusion was that these cultures can be grown in stirred-tank type bioreactors (10–13). However, under such conditions the cells produce only very small amounts of alkaloids. Recently, we found that by recirculation of the aeration gas in the bioreactor, similar production levels can be achieved as in shake flasks (28). Immobilization of *Ca. roseus* cells has been reported as a way to improve production (20,72,74). Hairy roots have also been found to produce ajmalicine and serpentine; however, growth rates of such cultures are very low (65, S.S. Hoekstra, J.H.C. Hoge, F. van Iren, V.R.L. Libbenga, and R. Verpoorte, unpublished results). Elicitation has been found to increase tryptamine and ajmalicine production (75).

The economic feasibility of the production of ajmalicine has been discussed. With a current estimated price of about $1500/kg, the productivity of the *Catharanthus* cell cultures is too low. A tenfold increase in productivity would, however, result in a competitive price. Below we will discuss current research aimed at improving the productivity.

Rauvolfia cell cultures have so far been shown to be one of the best producers of terpenoid indole alkaloids. The commercially interesting alkaloid ajmaline is produced in suspension cultures of several *Rauvolfia* species. The highest level (0.5% of dry wt) was reported for *Rauvolfia serpentina* cell cultures (76). Stöckigt and co-workers (77,78) studied alkaloid production of several *Rauvolfia* species. They found that the major alkaloid produced was raucaffricine, a glycoalkaloid which is an intermediate in ajmaline biosynthesis. In *R. serpentina* a production of 1.6 g/liter could be achieved for this glycoalkaloid when subcultured on an induction medium (79). Hydrolysis of the glycoside yielded vomilenine, a compound with several reactive groups and thus of interest as a synthon in biomimetic syntheses.

Reserpine and rescinnamine, the two other alkaloids presently isolated from *Rauvolfia* on an industrial scale, are only produced in small amounts by the cell cultures. The highest level reported was 0.1% of dry wt of *R. serpentina* suspension cultures, after cloning of selected yellow-green fluorescent cell aggregates (80).

The production of vincamine by *Vinca minor* cell suspension cultures has been patented (81). Levels as high as 3.3 g/liter were claimed. These cell cultures also produced 0.9 g/liter epivincamine. Alkaloids like ellipticine (82), tabersonine (83,84),

and camptothecine (85,86) have also been isolated from cell cultures but all at low levels (20).

Caffeine.—Although caffeine is a low-priced specialty chemical, much work has been done on its production in cell cultures. For cell suspension cultures of *Coffea arabica*, levels of 0.03–0.7% dry wt have been reported (87). The alkaloid is for a large part found in the medium. Usually also considerable amounts of theobromine are found (25–50% of total alkaloid). Alkaloids were also found to be produced when the cells were grown in a 14-liter stirred-tank fermenter (88). Immobilization of the cells in calcium alginate resulted in even higher productivities (88,89), reaching levels of 0.4 g/liter in 32 days.

Steroidal alkaloids.—The production of *Solanum* glycoalkaloids in cell cultures showed considerable variation. Zenk (90) reported for *Solanum laciniatum* cell clones levels of solasonine ranging from 0 to 3% of the dry wt, with a clear maximum at about 0.2%. In general the levels reported (20) are far below the levels of interest for these low-priced chemicals, used as raw material in steroid synthesis.

Miscellaneous.—Pharmaceutically important alkaloids such as physostigmine and pilocarpine have so far not been isolated from plant cell cultures.

TOWARDS IMPROVED PRODUCTIVITY.—Above we discussed the technological and economical feasibility of the production of natural products by means of plant cell cultures. The technology is feasible; the costs of the process proved to be such that only high-value specialty chemicals can be produced. Furthermore, we discussed the approaches generally applied to establish high producing cell lines that might meet the productivity requirements for a commercial process.

For alkaloids none of these approaches have so far resulted in a commercial process. Berberine is probably closest to that point.

Does that mean that we have reached the end of the road concerning the possibilities of a plant cell biotechnological production process for alkaloids? Certainly not!

A simple calculation can tell us that we are still far away from the full potential of production of secondary metabolites by plant cell cultures. If we assume that at a given moment we would be able to completely channelize the plant metabolism towards the biosynthesis of a certain product, in one doubling time of the cells the same amount of product as the weight of the biomass could be produced. For instance, at a biomass density of 50 g/liter we would produce 50 g/liter of product in about 24 h. This seems a high production, but, for example, in the production of penicillin similar levels do occur. Of course we will not be able to completely turn metabolism into one direction; however, for example the phenylalanine pathway has been reported to amount to as much as 30% of the total metabolism in plant cells. The high levels of secondary metabolites found in certain plant tissues are a further proof that plants are capable of considerable biosynthetic efforts. How will we be able to reach such levels? For that goal we have first of all to learn more about the regulation of product accumulation, in order eventually to be able to devise such methods as genetic engineering to improve the secondary metabolite production.

There are three factors affecting accumulation: (a) de novo biosynthesis of the product; (b) compartmentalized accumulation of the product; and (c) breakdown of the product. The actual production will be the result of biosynthesis minus breakdown (catabolism and chemical breakdown) of the product, provided that storage of the product is possible, thus avoiding autointoxication of the producing cells. We will discuss these three points in some more detail using some of the results of our own research as an example.

Biosynthesis.—In the past years a large research effort has been put into studies of the biosynthesis of natural products, using plant cell cultures as easy to handle model systems. In doing so, the complete pathway of the biosynthesis of benzophenanthridine alkaloids has been elucidated (91) and much work has been done on the biosynthesis of terpenoid indole alkaloids (for reviews see 92–96). Once the biosynthetic pathway involved is known and its enzymes are characterized and isolated, one can clone the genes. Subsequently the regulation of the secondary metabolism can be studied at the level of the genes. Eventually genetic engineering approaches can be considered to improve the production of secondary metabolites.

In our studies of the production of terpenoid indole alkaloids we have chosen *Catharanthus roseus* (Apocynaceae) as a model plant. Plants and cell cultures of some *Tabernaemontana* (Apocynaceae) and *Cinchona* (Rubiaceae) species were used for comparison of the findings in the *Ca. roseus* system with the regulation of similar pathways in related plants.

Three enzymes play a key role in the biosynthesis of the terpenoid indole alkaloids (Figure 3). Strictosidine synthase (SSS) couples the products of two different pathways, secologanin and tryptamine, to yield strictosidine, the universal precursor of all ca. 3000 known terpenoid indole alkaloids (97). The pathways leading to these two precur-

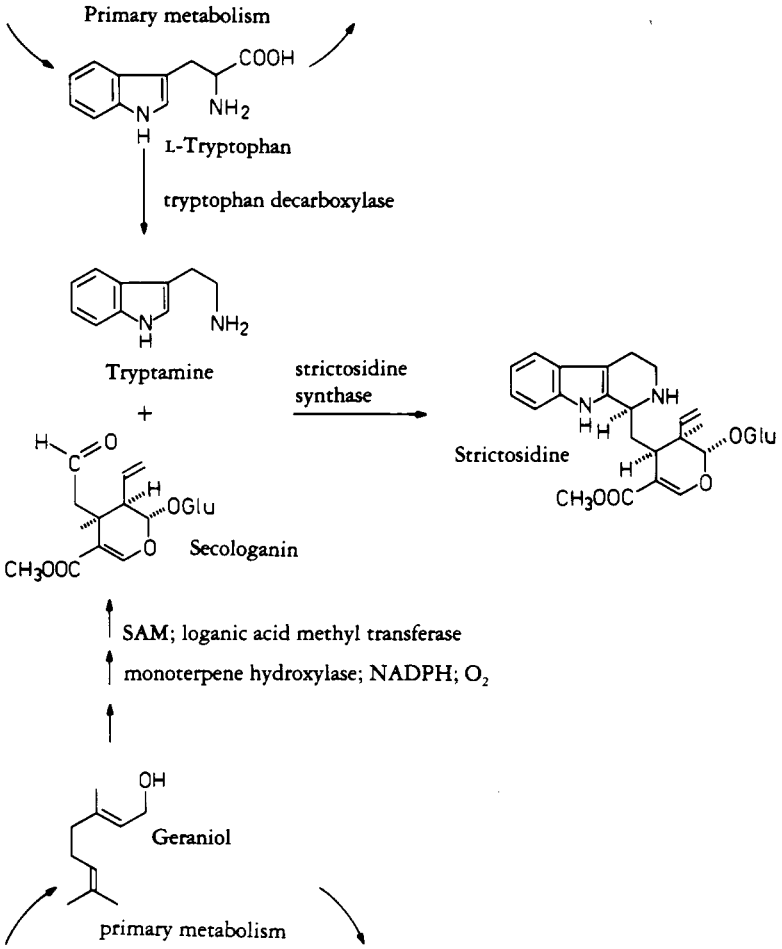


FIGURE 3. Early steps of terpenoid indole alkaloid biosynthesis.

sors are both derived from primary metabolism. The enzymes catalyzing the conversion of tryptophan and geraniol into, respectively, tryptamine [tryptophan decarboxylase (TDC)] and 10-hydroxygeraniol [geraniol-10-hydroxylase (G10H)], are both highly regulated (98,99). The latter enzyme seems to be a limiting factor; we observed several cell cultures containing large amounts of tryptamine but no alkaloids. These cultures did not show any G10H activity. The three enzymes mentioned, SSS, TDC, and G10H, were chosen as first targets for our studies.

Hplc assays for determining SSS (100) and TDC (101) activities were developed, allowing both the analysis of the substrates and the products. This avoided problems of conversion of the substrates by competing enzymes or further conversion of the products, both resulting in erroneous results. Subsequently, both enzymes have been purified. In the case of SSS, seven isoenzymes were isolated and characterized, all having similar properties, most likely differing in their degree of glycosylation (A. de Waal and R. Verpoorte, unpublished results). The enzyme TDC, which consists of two identical subunits, was found to contain two molecules of pyridoxal phosphate as cofactor. Moreover, two molecules of pyrroloquinolinequinone (PQQ) could be isolated per molecule of the dimeric enzyme (102). Antisera were raised against both SSS and TDC, and parts of their amino acid sequence were determined, thus opening the way for molecular biologists to clone the genes from cDNA expression libraries of *Ca. roseus* (103–105). Both enzymes proved to be encoded by single copy genes within the *Ca. roseus* genome. The sequence found for the *tdc* gene was identical to that reported by De Luca *et al.* (106). The *sss* gene showed extensive homology with the *sss* gene cloned from *Rauvolfia serpentina* (107).

With these genes available and their sequences known, further studies on their expression became possible. The *tdc* and *sss* genes were found to be coordinately expressed in *Ca. roseus*. In plants, their steady state mRNA levels are highest in roots, 5- to 10-fold lower in leaves, and very low in stems and flowers. In cell cultures, their transcription is repressed by auxins and induced by fungal elicitors. Subculturing on auxin-free media, for example, results in a rapid induction of the transcription of the *tdc* and *sss* genes, resulting in an increase of the respective mRNA levels and a subsequent increase of the activities of the encoded enzymes. Repression and induction occur rapidly and independent of de novo protein biosynthesis, suggesting that auxins and elicitors modulate the activity of pre-existing transcription factors (104,105).

Recently, candidate cDNA clones have been obtained for the third key enzyme, G10H, in terpenoid indole alkaloid biosynthesis, and this membrane-bound cytochrome P-450 monooxygenase was purified to homogeneity (108). Furthermore, a cDNA clone coding for the enzyme NADPH:cytochrome P-450 reductase has been isolated and shown to correspond to a single gene copy. This membrane-bound reductase is essential for any cytochrome P-450 catalyzed reaction, since it functions in electron transfer to P-450 enzymes. The gene has clear homology with reductase genes from animals and yeasts: in particular, the parts coding for the co-factor binding sites are highly conserved (A.H. Meijer, J.H.C. Hoge, and R. Verpoorte, submitted for publication).

The probes developed for the *sss* and *tdc* genes did not detect any mRNAs in *Cinchona* plant material. Therefore, in this plant species studies on the regulation of alkaloid biosynthesis were done on the level of alkaloid production and enzymes only (Figure 4). Neither quinoline alkaloids (such as quinine, quinidine, cinchonine, and cinchonidine) nor indole alkaloids (such as cinchophyllines and cinchonamine) are produced in cell suspension cultures; therefore plants and seedlings were used in these studies. In seeds of *Cinchona* no alkaloids are present, and neither SSS nor TDC activity can be detected. However, during germination, at the very moment the radicle is emerging from the seed, the activities of these enzymes rapidly increase, tryptophan

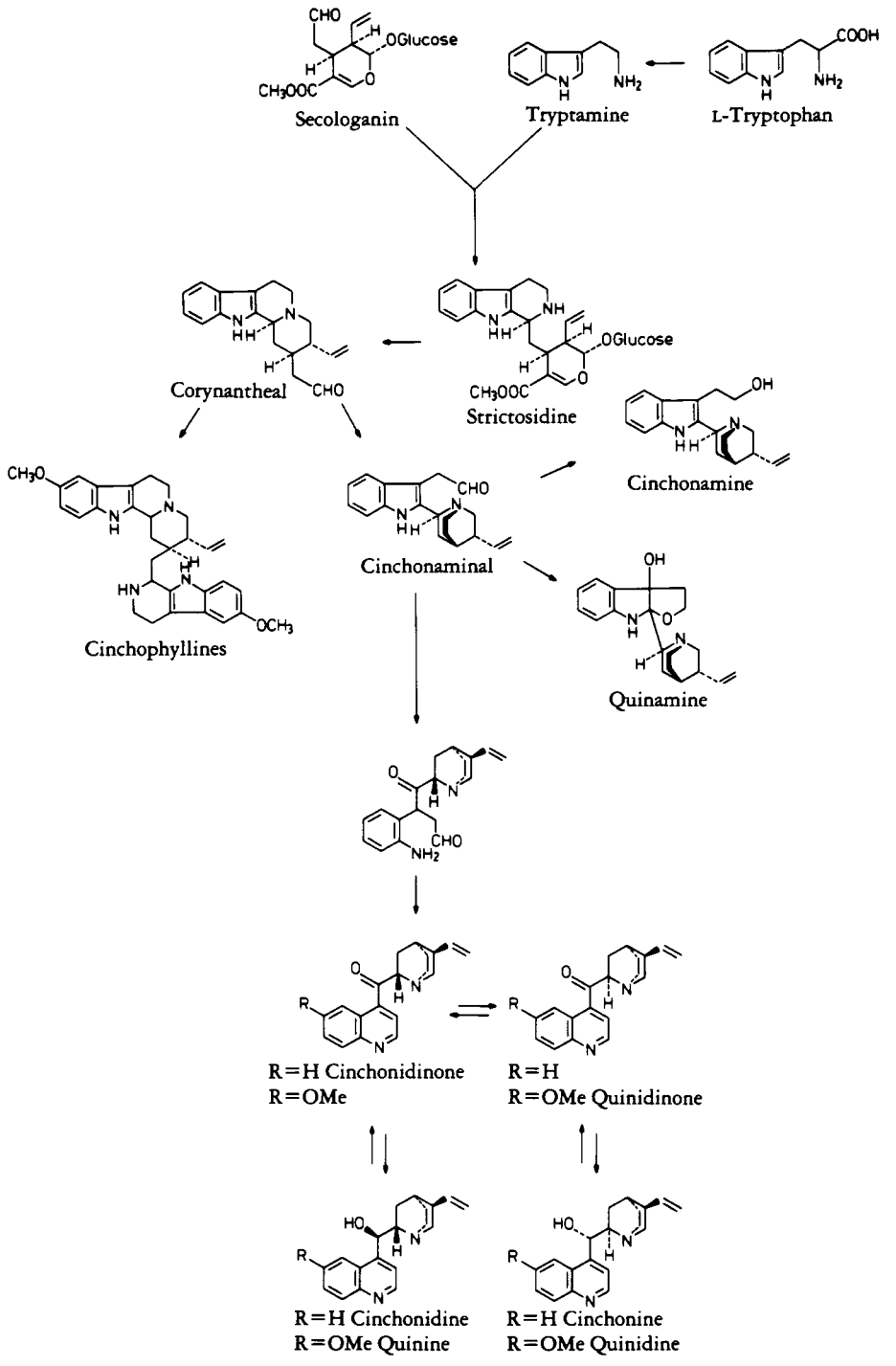


FIGURE 4. Biosynthesis of *Cinchona* alkaloids.

levels increase, and subsequently tryptamine and alkaloids are formed. In the first week all tryptophan is being converted into quinoline alkaloids. Apparently in the seeds all the precursors necessary to form the alkaloids are present, and during germination these are stoichiometrically converted into alkaloids. This conversion uses less than 0.5% of

the total energy consumed during the development of the seedling (109,110). Feeding of various precursors of the alkaloid biosynthesis does not disrupt the metabolic regulation: only at higher levels they become toxic for the seedlings (111). It was shown that the increase of quinoline alkaloid levels coincides with a strong antifeedant activity against slugs (110), the levels being just sufficient to provide a complete deterrence. This suggests a protective role of the quinoline alkaloids against various herbivores. In young leaves, however, mainly indole alkaloids are found. These alkaloids have little activity against the slugs but exhibit strong activity against among others *Spodoptera exicua* larvae which usually feed on leaves of trees. Their levels in young leaves are again such that complete feeding deterrence occurs. *Cinchona* plants thus have developed a protection against different herbivores on the basis of one pathway starting with strictosidine. In the seedlings and the lower parts of the plant, including the roots, the quinoline alkaloids are produced as protectants, in the leaves the indole alkaloids.

In cell suspension cultures of *Cinchona* species no alkaloids are produced, and neither TDC nor G10H activity can be found in such cultures. However, SSS activity was found to be transiently present (112). From *Cinchona robusta*, SSS was purified (113); in fact four isoforms were found to be present, two with a pI of 6.5 (IA and b) and two with a pI of 7.5 (IIa and b). In each group one enzyme had a mol wt of about 33.000 and one of about 35.000, estimated from gel electrophoresis. By means of laser desorption ms, molecular weights of three of these isoforms were determined as 35.895 (Ia), 35.814 (IIa), and 31.310 (IIb). All enzymes are glycosylated. Not only do the pI values differ from those of the SSS isoforms isolated from *Ca. roseus*, but also the substrate specificity is different. In contrast with the *Ca. roseus* enzyme, the *Cinchona* enzymes accept also 5-methoxytryptamine as substrate, though with lower efficiency than tryptamine. This points to the possibility that the methoxy group in quinine and quinidine might already be introduced at a very early stage of the biosynthetic pathway. Interestingly, SSS was found to be strongly inhibited by quinine (3.3 mM, complete inhibition). The conclusion must be drawn that SSS [a vacuolar enzyme in *Ca. roseus* (114)] and quinine (stored in vacuoles) cannot be located in the same compartment (see below).

In *Ca. roseus* the alkaloid biosynthesis is induced by fungal elicitors. Similar experiments in *Tabernaemontana divaricata* cell cultures showed that alkaloid production is blocked by such a treatment. Instead a triterpenoid pathway is induced, resulting in the production of considerable amounts of ursolic acid and dulcioic acid derivatives (115). Apparently, the pathways leading from tryptophan and geraniol can be regulated quite differently, serving the plant's needs in different ways.

With some of the genes cloned, plant genetic engineering experiments can be attempted in order to eventually improve production. To first prove the feasibility of the expression of the isolated genes into actual active enzymes, the *tdc* gene has been linked to the constitutive CAMV 35S promoter and introduced into tobacco, a plant which normally does not contain this enzyme. This resulted in healthy plants producing about 1% (of dry wt) tryptamine (103,116), i.e., levels commonly found for indole alkaloids in plants. Apparently plants can produce these secondary metabolites from normal primary metabolism, without negatively interfering with plant growth and development. McKnight *et al.* (114) introduced a CAMV35 promoter-*sss* gene construct into tobacco plants and found high levels of the enzyme in the plants. The enzyme was found to be present in the vacuoles. The *Rauvolfia sss* gene has been expressed in *Escherichia coli*, resulting in cultures which had a 20 times higher enzyme activity per liter than the plant cell cultures (117). This gene has also been expressed in yeast and insect cells (91).

The introduction of the *tdc* gene in an antisense sequence into *Ca. roseus* callus cultures resulted in an inhibition of alkaloid production (103).

It is thus feasible to isolate genes from secondary metabolite biosynthesis and, combined with strong promoters, to reintroduce them in plants, where they result in the production of active enzymes and may increase or alter secondary metabolite production. The introduction of the ornithine decarboxylase gene from yeast into *Nicotiana rustica* hairy roots resulted in a twofold increase in nicotine production (118). This shows that also genes from other sources may be considered for genetic engineering of secondary metabolism.

Storage compartment.—So far little is known about the compartmentation of terpenoid indole alkaloid biosynthesis and accumulation. In isolated vacuoles of *Ca. roseus* we were able to detect SSS (T.J.M. Blom, L.H. Stevens, and R. Verpoorte, in preparation), whereas TDC could not be detected in these vacuoles, thus further supporting the idea that this is a cytosolic enzyme.

Chorismic acid is the precursor for the aromatic amino acids phenylalanine, tyrosine, and tryptophan. These amino acids are precursors for numerous secondary metabolites. It has been postulated that for the biosynthesis of these amino acids two separate pathways exist, a cytosolic and a plastidial, respectively involved in secondary and primary metabolism (119,120). However, in our studies on the enzyme anthranilate synthase (AS), catalyzing the first step of the biosynthesis of tryptophan from chorismic acid, so far no evidence has been found for the presence of two separate pathways for the tryptophan biosynthesis. Also the high levels of tryptamine found in *tdc*-gene-containing transgenic tobacco plants (see above) support the idea that in indole-alkaloid-producing plants tryptophan is coming from normal primary metabolism and thus probably is biosynthesized in plastids. The same site has been postulated for the biosynthesis of C_{10} terpenoids. Where the iridoids are synthesized is not known.

The strong inhibition of the enzyme SSS by quinine in *Cinchona robusta* points to different compartments for these two components. As both are supposed to be present in the vacuole, quinine might after its production be stored in vacuoles of cells that no longer biosynthesize strictosidine. Transport of the alkaloids through cell membranes has been shown to be quite rapid (121–125); separate cells for storage and production are therefore conceivable. Different models have been postulated for the mechanism of accumulation of alkaloids in vacuoles. For example, active transport by selective carrier proteins (126,127) and an ion-trap mechanism (128) have been proposed for ajmalicine. Blom and co-workers (122,124,125) showed that an ion-trap mechanism is active in combination with a chemical trap, converting ajmalicine into serpentine (Figure 5). This oxidation by basic peroxidases present in the vacuoles results in a product that cannot pass the vacuole membrane.

One of the constraints in the accumulation of terpenoid indole alkaloids might be their low solubility in H_2O, even under acidic conditions. The better solubility of compounds like the glycoalkaloid raucaffricine may thus be one of the reasons that this alkaloid is accumulated to high levels. In a cell line with a high productivity it might be necessary to provide an external storage site for the alkaloids, as the solubility of the alkaloid in the vacuole will otherwise be a limiting factor in production. Further studies are necessary to unravel the role of compartmentalization in the regulation of alkaloid biosynthesis and accumulation.

Breakdown.—Little attention has been paid to the role of catabolic processes in plant cell cultures. That such processes do occur can easily be concluded from a comparison of the levels of alkaloids in plant cells shortly after subculturing with the levels just before subculturing. Often the levels shortly after subculturing are considerably lower. Also in some cell cultures of *Tabernaemontana* species, it was noted that a rapid decrease of alkaloids occurred in the stationary phase (Figure 6) (129). Further studies

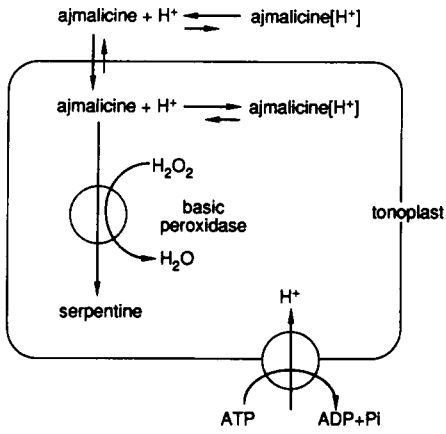


FIGURE 5. Model of accumulation of ajmalicine and serpentine in cells of *Catharanthus roseus* (124,125).

on these cultures using ^{15}N-labelled alkaloids, obtained by feeding labelled nitrate and ammonia to cell cultures, showed that considerable breakdown of the alkaloids occurred, and at the end of the growth phase, beginning stationary phase, breakdown equals the biosynthesis (Figure 7) (130). In *Ca. roseus* cell cultures, added ajmalicine is

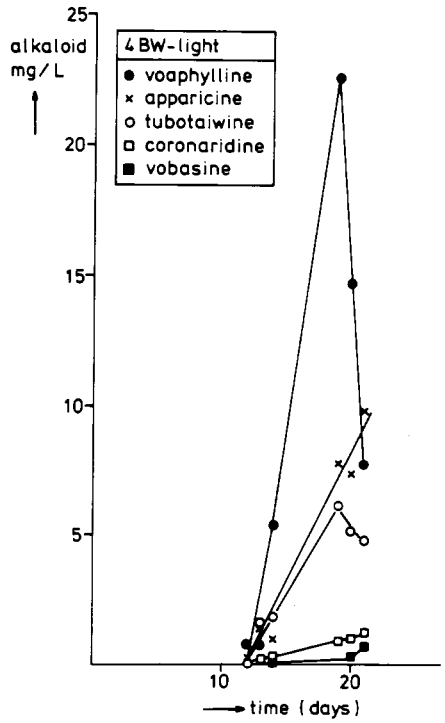


FIGURE 6. Alkaloid accumulation in plant cell suspension of *Tabernaemontana divaricata* (129).

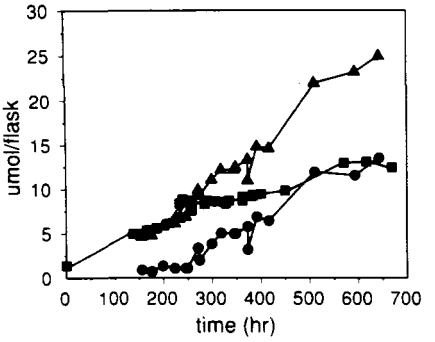


FIGURE 7. De novo biosynthesis (▲), accumulation (■), and breakdown (●) of *O*-acetylvallesamine in plant cell suspension of *Tabernaemontana divaricata* (130).

catabolized (R. dos Santos, J. Schripsema, and R. Verpoorte, in preparation). Using deuterium-labelled ajmalicine it was shown that breakdown at the end of the production phase equals biosynthesis. Further studies will be necessary to identify the cause of the breakdown, i.e., chemical decomposition or enzymatic catabolism, and to learn whether compartmentalization (e.g., the presence of storage sites) may reduce the rate of the degradation, thereby increasing the productivity.

CONCLUSIONS

Presently no commercial production of alkaloids by means of plant cell cultures exists. The technology is feasible. The major task will be to improve alkaloid levels in the cell cultures. However, our knowledge about the regulation of secondary metabolism is still very limited. From the results concerning biosynthesis, storage, and catabolism of the terpenoid indole alkaloids, it is clear that some interesting possibilities to increase production are still to be explored. The regulation of biosynthesis on the level of the genes is just one part of the total regulation concerning secondary metabolism. Regulation mechanisms on the level of enzymes and products (e.g., transport, storage, catabolism) do also play a major role. Future studies should thus combine the efforts of phytochemistry (including enzymology), cell biology, plant physiology, and molecular biology to unravel the regulation of secondary metabolite production on all its levels. Such studies will not only contribute to solving problems concerning the production of secondary metabolites by cell cultures (or plants) for industrial purposes. They will also shed light on the role of secondary metabolism in plant survival, and thus may be helpful in the future to obtain more resistant plants. For such studies collaboration with plant ecologists will be necessary.

THE FUTURE.—How will things for secondary metabolites look 50 years from now? This is not an easy question to answer, since plant genetic engineering is only about 20 years old and metabolic engineering is just in its infancy. But we might try to make some predictions.

New natural products from rare plants, which are just as crucial as penicilline (e.g., antitumor or anti-HIV products), might be found and be produced by plant cell cultures.

Genetic engineering will develop in some sort of molecular surgery, and using these

methods plants will have been transformed with sense or antisense genes from secondary metabolite pathways, resulting in:

plants that make complete new products useful as specialty chemicals;
new, easy to culture, plants that make known specialty chemicals in higher yields (e.g. *Cinchona* alkaloids in *Ca. roseus*);
plants with increased resistance against diseases or herbivores due to an altered secondary metabolism;
plants with new colors of the flowers or fruits;
plants with improved taste or smell;
plants devoid of toxic secondary metabolites (e.g., *Solanum* glycoalkaloids);
plants devoid of unwanted secondary metabolites (e.g., caffeine in coffee beans).

It is easy to predict that these results can only be achieved by close collaboration of different disciplines, as no single discipline will be able to solve the encountered problems alone.

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