Plant Cell, Tissue and Organ Culture 18: 231–280, 1989

Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: a literature survey

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Key words: *Catharanthus*, cell culture, indole alkaloid, review, tissue culture

Abstract. The literature concerning the formation of secondary metabolites in cell and tissue cultures of *Catharanthus roseus* has been reviewed. Several aspects involved in the formation of secondary metabolites are discussed; e.g. regulation of secondary metabolism, environmental factors influencing secondary metabolism, biosynthesis and enzymology of the products, analysis of product formation, immobilization of cultured cells and stability of cell lines. Some economical aspects of production processes are discussed.

Introduction

Due to the presence of the therapeutically valuable cytotoxic alkaloids vinblastine and vincristine in *Catharanthus roseus*, this plant became one of the major fields of interest in modern plant cell biotechnology. The low yields of these dimeric indole alkaloids (approx. 0.0005%) and the subsequent high price of them were major motives to study the possibilities for the production of these alkaloids by cell and tissue cultures. The first efforts in this field date from the sixties. Carew [1] reported the first prolonged growth of a suspension culture after earlier reports by other researchers on crown gall and callus cultures (reviewed in [2]). Since 1966 the knowledge of and the insight in the alkaloid or, in general, secondary metabolite production processes has deepened enormously. At present, however, it is still not possible to produce these dimeric indole alkaloids by suspension cultures, which is considered to be the most suitable culture system for industrial purposes.

Catharanthus roseus (L.) G.Don or periwinkle originates from Madagascar and belongs to the family of the Apocynaceae. The plant has been

thoroughly investigated for secondary metabolites, which led to the isolation of approximately 90 indole alkaloids. The individual alkaloids possess, besides the antitumor activity of vincristine and vinblastine, a wide spectrum of biological effects [3, 4]. For instance ajmalicine (raubasine) is used in the treatment of hypertension and obstructive circulatory diseases. The pharmacology of the dimeric indole alkaloids vinblastine and vincristine has been investigated in detail [5].

Also from other genera of the family Apocynaceae like *Voacanga* and *Tabernaemontana* many indole alkaloids have been isolated. The production of secondary metabolites in cell and tissue cultures from plants belonging to the tribe Tabernaemontaneae has recently been reviewed [6].

Various aspects of the production of useful metabolites by plant tissue cultures have been extensively discussed in a number of reviews [7–22].

In this review we try to summarize the increasing knowledge on the growth and production of secondary metabolites by cell and tissue cultures of *C. roseus*. During the last two decades, several hundred publications have appeared on this subject, which made us to restrict ourselves to the regulation of secondary metabolite formation influenced by developmental and environmental factors.

In Chapter 1 the secondary metabolites which have been isolated from cell and tissue cultures of *C. roseus* are described. The production of these metabolites can be regarded as the result of the interaction of environmental conditions and the genotype of the cultured plant cells. The environmental conditions, like culture medium, light and temperature, control the metabolism, growth and differentiation of the plant cells. Evidence is accumulating that production of secondary metabolites and cell morphological differentiation are indissolubly connected. Chapter 2 describes the relation between plant material or culture type and product yield. The environmental factors influencing secondary metabolism, in particular the production of indole alkaloids in cultured *Catharanthus roseus* cells will be discussed in Chapter 3. In the subsequent chapters, regulation of the secondary metabolism (Chapter 4), biosynthesis and the enzymology of the products (Chapter 5), analysis and stability of product formation (Chapter 6 and 7, respectively), immobilization of plant cells (Chapter 8) and economical aspects of the production process (Chapter 9) are summarized.

This review covers the literature up to February 1988.

1 Secondary metabolites from cell and tissue cultures of *Catharanthus roseus*

Over the years a great number of secondary metabolites have been isolated

Table 1. Alkaloids isolated from *C. roseus* cell and tissue cultures.

Class/alkaloid	Type of culture[a]	References
Vincosan		
strictosidine	s	23, 24
strictosidine-lactam	s,sh	23, 25–27, 36
Corynanthean		
ajmalicine	c,s,sh,r	23–37, 39–42
ajmalicine, 3-epi-	c,s	28, 30–32, 34
ajmalicine, 3-epi-19-epi	s	28
ajmalicine, 7-hydroxy-indolenine	c	30, 31, 34
ajmalicine, pseudo-indoxyl	c	30, 31, 34
akuammigine	s	28
akuammiline	s	28
akuammiline, 10-hydroxy-desacetyl-	c	30–32, 34
akuammiline, desacetyl-	c	30–32, 34
alstonine	c	37
antirhine	s	28
cathindine	s	37
cavincidine	c,s	37
cavincine	c,sh	37, 44
cyclolochnerine, 21-hydroxy-	s	38
isositsirikine	c,s	23, 25, 28, 35
isositsirikine, 16R-19,20-E-	s	38
isositsirikine, 16R-19,20-Z-	s	38
mitraphylline	c	30, 37
perivine/perosine	c	37
pleiocarpamine	s	28
serpentine	c,s,sh	29, 33, 39, 43
sitsirikine	c,s,sh	28, 33, 37
sitsirikine, dihydro-	c,s	28, 37
tetrahydroalstonine	c,s,sh	28–31, 33, 34
yohimbine	c,s	23, 25, 28, 35
Vallesiachotaman		
vallesiachotamine	c,s	23, 25–28, 30, 31, 35, 40
isovallesiachotamine	c,s	26–28, 30, 31
Strychnan		
akuammicine	c,s,sh	24, 29, 37, 40, 44
akuammicine, 12-hydroxy-	s	29
lochneridine	c,s	37
943-strychnan-glycoside	s	35
Aspidospermatan		
tubotaiwine	c	30, 31

Table 1 continued.

Class/alkaloid	Type of culture[a]	References
Plumeran		
hörhammericine	s,sh	23, 25–29, 35, 36, 40
hörhammerinine	s,sh	23, 25–27, 29, 35, 36, 40
lochnericine	s	23, 25, 28, 29, 40
lochnerinine	s	23
minovincinine	s	28
tabersonine	c,s	28–32
tabersonine, 19-hydroxy	s	28
tabersonine, 19-acetoxy-11-hydroxy	s	25, 35
tabersonine, 19-acetoxy-11-methoxy	s	25, 35
tabersonine, 19-hydroxy-11-methoxy	s	35
vindoline	sh,cg	33, 36, 42
vindolinine	s,sh	23, 25–29, 35, 36, 40
vindolinine-N_b-oxide	s	28
vindolinine, 19-epi-	s	23, 25–27, 28, 29, 35
vindolinine-N_b-oxide, 19-epi-	s	28
Ibogan		
catharanthine	s,sh,r	24, 26–29, 36, 42
Bisindoles		
3′,4′-anhydro-vinblastine	sh	42
leurosine	sh	42
catharine	sh	42
vinblastine	c,cr,sh	45, 46, 47

[a] Culture type: c = callus, s = suspension, sh = shoot, r = root culture, cr = callus with root formation, cg = crown gall

Table 2. Other metabolites from cell and tissue cultures of *C. roseus.*

Tryptophan derivatives	tryptamine	several authors
	tryptamine, N,N-dimethyl-	25
	tryptamine, N-acetyl-	48
Phenolics		49
Anthocyanins[a]	petunidin	49
	malvidin	49
	hirsutidin	49
Fatty acids	oleic acid	48
	linoleic acid	48

[a] Only the aglycones have been identified.

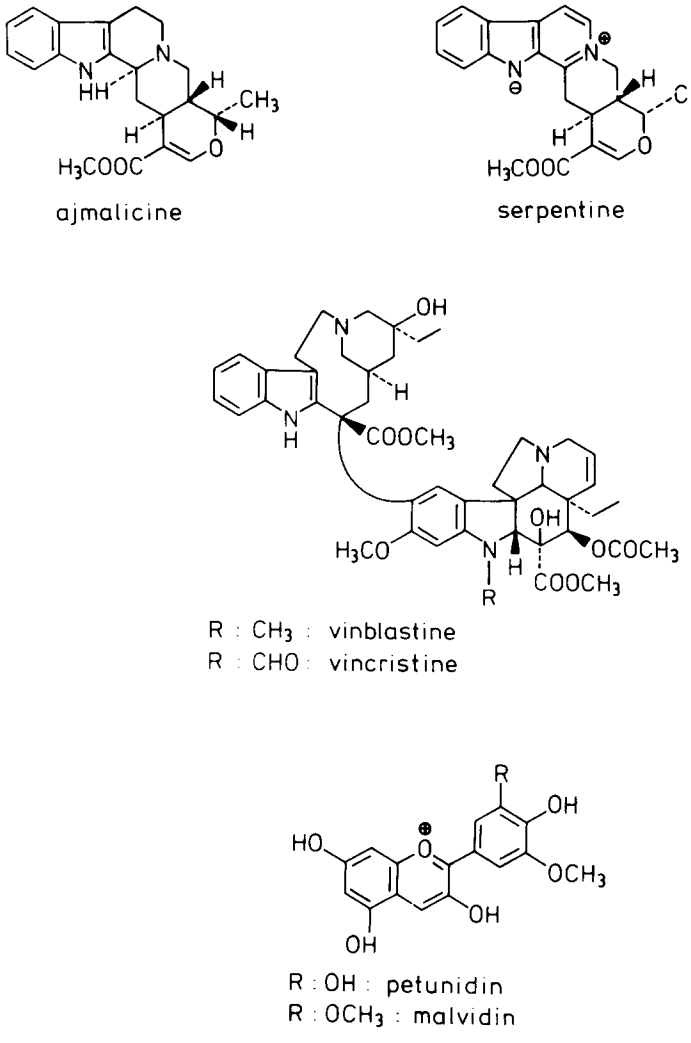
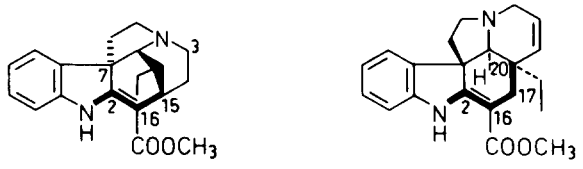


Fig. 1. Structure of ajmalicine, serpentine, vinblastine and two anthocyanidins.

from cell and tissue cultures of *C. roseus*. Besides the metabolites of main interest, the indole alkaloids, some anthocyanins have been isolated. The isolations are summarized in Tables 1 and 2 and some structures are presented in Fig. 1.

The alkaloids are classified according to Kisakurek & Hesse [50] and van Beek & van Gessel [51]. In contrast to others they distinguish in addition to an aspidospermatan class a plumeran class of alkaloids. The structural characteristics of these classes are the presence of a C(2)-C(16)-C(15) unit and the absence of a C(3)-C(7) bond for the aspidospermatan class, and a



aspidospermatan-type
tubotaiwine

plumeran-type
tabersonine

Fig. 2. Examples of an aspidospermatan and a plumeran alkaloid.

C(2)-C(16)-C(17)-C(20) unit for the plumeran class (Fig. 2). We therefore classified vindoline as a plumeran alkaloid. However, in all literature examined for this review, no distinction was made between these types and they were all classified in the aspidospermatan type.

Ajmalicine and serpentine, belonging to the large group of corynanthean alkaloids, are commonly found metabolites in cell and tissue cultures of *C. roseus*. The ibogan class is represented by catharanthine, which forms together with the plumeran alkaloid vindoline the basic skeleton of the dimeric indole alkaloids vinblastine and vincristine. Vindoline has not been isolated from undifferentiated systems and suspension cultures, for its biosynthesis differentiation is essential (see Chapter 3). Dimeric indole alkaloids may be isolated from a more differentiated callus culture [45] or organ culture [46, 47].

Besides the alkaloids, the anthocyanins form a second group of metabolites. The anthocyanins are often responsible for coloration of the cultures. Only some aglucons (anthocyanidins) have, so far, been identified.

2 Influence of plant material and culture type on product formation

The choice of the plant material and the culture type initiated thereof offers a wide spectrum of possibilities to influence the secondary metabolism. Table 3 summarizes different levels for a screening or selection procedure to obtain (high) producing cultures.

The genus *Catharanthus* comprises several *species* which are able to produce indole alkaloids [52]. In tissue culture studies the species *C. roseus* is commonly used. Stöckigt & Soll [29] isolated from a *C. ovalis* suspension culture e.g. apparicine, an alkaloid which has, so far, not been isolated from *C. roseus* cell and tissue cultures.

Kurz et al. [53] investigated over 2000 cell lines of three *C. roseus cultivars* for their variation in respect to physiological behaviour and biosynthetical

Table 3. Levels for screening and selection procedures to obtain alkaloid-producing cultures of *Catharanthus*.

Screening of different *species*, e.g. *C. roseus, C. ovalis, C. lanceus, C. pusillus, C. trichophyllus* etc.
Screening of different *cultivars*, e.g. *C. roseus* cv. Roseus, cv. Little Delicata
Screening of different *specimens* belong to the same species/cultivar
Screening of different *cell lines*: — initiated from identical plant parts — initiated from different plant parts — derived from the same cell clone (cell/protoplast)
Screening on different levels of *differentiation*: — plant — seedling — shoot, root culture — callus — suspension
Screening on *physiological* status: — heterotrophy/autotrophy — prototrophic (habituated)/growth regulator dependent — ha-/di-/polyploid
Screening on *genetical* status: — Ti-plasmid transformed — introduction of RNA/DNA by other techniques
Selection e.g. 5-methyltryptophan resistancy

capabilities. Between these three cultivars, differences were observed in production profiles. Of the cell lines (n = 458) initiated from cultivar Roseus, 16% produced corynanthean (C) together with aspidospermatan (A, including plumeran) type of alkaloids, 10% produced only strychnan (S) type of alkaloids, another 10% produced S together with A alkaloids. However, 32% of the cell lines were non-producers. Of the cell lines of cv. Roseus, 25% produced only one type of alkaloid. Altogether 8 different combinations of the production of C, S, A and ibogan (I) alkaloids were observed. Of the cell lines initiated from cultivar Little Delicata (n = 93), 45% produced a mixture of C, S, A and I alkaloids. Only 3% was non-producer. These cell lines frequently contained catharanthine, in relatively large amounts. A combination of C and A alkaloids occurred most frequently in the 85 investigated cell lines of cultivar Polka Dot. 6% was non-producer.

All the cell lines of the different cultivars were initiated from identical plant parts, namely from excised anthers of 1.5–2.0 mm buds of young plants. Alkaloid production ranged from 0.1–1.5% of cell dry weight.

Constabel et al. [54] screened 76 cell lines initiated from one *leaf* of a periwinkle plant. 62% of the cell lines produced a combination of C, S and A alkaloids. Variation of the alkaloid spectra was low when compared to that found with cell lines derived from different plants.

Zenk et al. [41] selected low and high-yielding *plants*. In cultures derived from the high-yielding plants the average alkaloid production was 3.7 times as much as in the cell cultures derived from the low-yielding plants. After a selection procedure at cell level, a cell culture was obtained which produced 1.5 times as much ajmalicine and serpentine as the original plant.

Petiard et al. [55] studied variability in a *strain* which had been stable for 8 years in chlorophyll content, growth rate and alkaloid production. Clones, 18-month-old, derived from protoplasts of the stable cell line, were distributed in two populations. One was chlorophyllous and had a high diversity of alkaloids, while the other was non-chlorophyllous and had a small diversity of alkaloids. No clone produced more alkaloids than the mother strain. The origin of the variation was probably epigenetic and the cloning procedure was selective for some cell types with the risk of losing interesting cell lines.

Cresswell [56] selected over 100 cell aggregates of less than 45 cells with a strong autofluorescence from a plated suspension culture. Only 9 aggregates could be raised to a suspension culture: three of these cell lines produced alkaloids, one of them produced more ajmalicine and serpentine than the parent line.

Screening on cell and protoplast level can be performed by using flow cytometry. Brown et al. [57, 58] sorted cells with a high content of the strongly fluorescent alkaloid serpentine. Using microfluorimetry, a positive correlation was detected between vacuolar pH and serpentine content.

Selection methodology in general with emphasis on cell aggregate and single-cell cloning has been described by Yamada [59]. Spontaneously obtained and induced variability has been reviewed by Courtois [60] and Rideau [61], respectively.

The methods described above show possible strategies to obtain stable and high-producing cell lines. Screening on the level of morphological *differentiation*, e.g. organ cultures, can be useful if the metabolites to be studied are not produced in undifferentiated cultures. The biosynthesis of the metabolites might require some differentiation, e.g. vinblastine can be isolated from callus cultures but not from suspension cultures; the vindoline biosynthesis is developmentally regulated (see Chapter 3). On the other hand it might be expected that the less differentiated systems produce metabolites which could not be isolated from well-differentiated systems. Endo et al. [42] compared the alkaloid contents of intact plants with that of

a shoot, a root and a cell suspension culture. The alkaloid pattern in root and suspension cultures was similar to that of roots from intact plants. The content of the shoot cultures was similar to that found in leaves and stems of intact plants; however, vinblastine and vincristine could not be detected in the shoot cultures. It was suggested that the different types of alkaloids were regulated by different factors. Catharanthine, as a representative of the ibogan alkaloids, was consistently found in all tissues; in dark-grown suspension cultures, higher contents were found than in any other (organized) tissues. The content of ajmalicine, belonging to the corynanthean type of alkaloids, was closely associated with light treatment. The relatively low levels of ajmalicine in leaves and stems of intact plants, compared with the levels in the roots, was suggested to be due to the sun light, rather than morphological differences. The biosynthesis of vindoline, a plumeran type of alkaloid, seemed to be dependent on both light and organogenesis. Upon transfer of light-grown shoot cultures to the dark, vindoline levels decrease drastically. Dimeric alkaloids occurred only in the tissues where both vindoline and catharanthine were present [42].

An attempt to induce cell differentiaiton chemically by the nucleoside analogue 5-azacytidine resulted, besides the normal alkaloid profile, in the formation of a new metabolite: lirio-resinol B mono-β-D-glycoside [62].

Another possibility to influence the secondary metabolism is a changed *physiological* status. Tyler et al. [63] induced photoautotrophy in suspension cultures of periwinkle, however, no vindoline or dimeric indole alkaloids could be isolated from these cultures.

Leaves of polyploid plants contained commercially interesting amounts of vinblastine [64]. Although some remarks were made on polyploidy in cell cultures [53, 65], there is no clear correlation with alkaloid production capacity. Polyploidy occurs also spontaneously after subculturing. Suspension-cultured cells investigated during the first 10 days of growth were mainly diploid ($n = 16$) with a few cells ($< 5\%$) tetraploid and a smaller number ($\ll 1\%$) with $n = 15$ or $n = 31$ [65].

Prototrophy (the phenomenon that a culture produces enough growth regulators to support its own growth) of the cultures can be obtained by habituation or genetic transformation by *Agrobacterium* infection. Both possibilities have been compared recently by Eilert et al. [66], whereby both culture types were able to produce alkaloids; neither habituated nor tumorous cell suspension cultures accumulated vindoline. From crown-gall tissue, vindoline could be isolated (Table 1). Hairy root cultures, obtained by transformation with *A. rhizogenes*, produced vinca alkaloids [67]. Changes in *genetic* constitution can also be obtained by the introduction of foreign DNA/RNA by e.g. electroporation [68].

Both Scott et al. [69] and Schallenberg & Berlin [70] tried to obtain high-producing cell lines by *selecting* 5-methyl-tryptophan-resistant cell lines. Although the free tryptophan levels within the cells were 30–40 times higher, it did neither result in an increased accumulation of tryptamine nor of indole alkaloids. Cresswell [56] and Stafford & Smith [71] obtained similar results with 5-methyltryptophan-resistant cell lines. Sasse et al. [72] selected cell lines with increased tryptophan decarboxylase activity by using, among other things, 4-methyltryptophan resistancy (4-methyltryptophan is, in contrast to 5-methyltryptophan, decarboxylated by tryptophan decarboxylase). Resistant cell lines showed higher accumulation of tryptamine (3 to 5-fold) and although most cell lines were non-producers, one cell line accumulated more ajmalicine on normal growth medium than obtained by inducing the original cell line.

3 Environmental factors influencing accumulation of secondary metabolites

3.1 Medium

In early days of research in plant cell biotechnology, culture media were developed for maintaining the cultures, i.e. the media should be able to induce and maintain the growth of the cells. This research led to the development of several media, e.g. B5 [73] and MS [74]. Later on, when research focused more on the production of useful chemicals by plant cell and tissue culture, still the same media were used, which usually resulted in low yields of the desired products. One then started to optimize the media for production purposes. Many constituents of the culture media were tested for their influence on the production of the desired secondary metabolites. For *Catharanthus* the optimization of the medium constituents resulted in two types of media. The alkaloid production medium, developed by Zenk et al. [41] increased alkaloid yields remarkably by lowering the amounts of nitrogen, phosphate and growth regulators. Also, this medium contained an increased amount of sucrose, and the alkaloid precursor tryptophan was added. The second medium type, developed by Knobloch & Berlin [39], gives rise to a more or less general protein synthesis induction, evoked by high sucrose concentrations. A disadvantage of both types of media is that they can only be used in a two-stage production process. The first stage comprises the growth of biomass on the standard culture media, while in the second stage, alkaloid production is elicited by transferring the cells into the production or induction medium. These media are not suited for subculturing, although the alkaloid production medium gives rise to a reasonable

Table 4. Growth and alkaloid production by cell suspensions of *C. roseus* on different media [after 65].

Medium	Biomass yield (g dry wt l^{-1})	Rate of alkaloid accumulation (mg l^{-1} day^{-1})		Number of other alkaloids detected
		serpentine	ajmalicine	
B5-control [73]	8.5	0.03	0	0
Induction [39]	2.0	0.04	0	4
Production [41]	10.3	3.2	0.7	10
M3 [65]	10.0	6.2	4.1	21

increase in biomass. Morris [65, 75] perceived this problem and composed a medium which could be used for subculturing and at the same time resulted in a high alkaloid yield. He succeeded by optimization of growth regulators in a standard MS medium. Compared with the induction and production medium, alkaloid production was superior in the combined growth-production medium (Table 4).

Similar results were also obtained by Smith et al. [76]; optimization of NAA and kinetin increased catharanthine yields. Accumulation could further be increased by the inclusion of lactose as carbohydrate source and the addition of abscisic acid or sodium chloride.

In the following subsections the uptake and the influence of the various medium constituents on growth and production of secondary metabolites (alkaloids, anthocyanins and phenolics) will be discussed. Various strategies designed to optimize alkaloid production were compared by Berlin et al. [77].

3.1.1 Carbohydrates

Besides water, the carbohydrate usually is the main constituent of the medium. It serves as carbon and energy source in the non-photosynthetically active cultures. Sucrose is the carbon source of choice of many research groups. Increased concentrations led to an increase in alkaloid production [41, 65, 78]. In culture media, it is hydrolysed into glucose and fructose; their exhaustion results (usually) in the beginning of the stationary phase (see e.g. [78]). Our own experiments indicate also an uptake of sucrose as such (unpublished data). Increasing the initial sucrose concentration from 2% to 6% resulted in increased ajmalicine and serpentine accumulation (9- and 4-fold, respectively), while the intracellular tryptamine concentration was related to alkaloid production [79]. Although one negative effect of increased initial sucrose concentrations on alkaloid production has been reported [80], at present it is a commonly accepted phenomenon that in-

creased sucrose concentrations improve alkaloid yields; partly due to an increased biomass yield. Thus, the alkaloid production medium [41] contains 5% sucrose.

Knobloch & Berlin [39, 81] obtained high ajmalicine contents by transferring 2-week-old cell suspension cultures to a 10-fold volume of an 8% sucrose solution ('induction medium'). The alkaloid accumulation started two days after the transfer and reached a constant level after ten days. Furthermore, an enhanced level of phenolic compounds was found, whereas the growth of the culture was low. Sucrose at 8% was found to be the optimal concentration in the range of 4–12% tested. Equimolar concentrations of glucose or maltose resulted in similar production yields, equimolar concentrations of fructose, galactose or lactose yielded only about half of that obtained with 8% sucrose. In contrast with these results, Smith et al. [76] observed increased biomass and catharanthine yields with lactose as carbohydrate source.

Carbohydrate limitation influenced several enzyme activities within the primary metabolism, however, cell viability remained at a high level for several days after limitation [82].

3.1.2 Nitrogen

Nitrate and ammonium salts are commonly used as nitrogen source in plant cell and tissue culture media. Addition of both ions results in a rapid increase of biomass, however, production formation (alkaloid, anthocyanins and phenolics) is inhibited. Product accumulation is strongly enhanced by reducing or deleting mineral nitrogen [39, 41, 49]. The ratio between the amount of nitrate-N and ammonium-N is often a characteristic for culture media. E.g. for MS medium [74] the nitrate/ammonium ratio is 2 and for B5 medium [73] it is 12. Slywka & Krueger [83] reported only a mild qualitative and quantitative influence on alkaloid production. However, when the nitrate/ammonium ratio was < 1, growth was not supported. Both ions are taken up during the growth phase at more or less equal rates; ammonium, usually the minor component, is exhausted as the first one [78, 79, 84]. It might be concluded that the limitation of nitrogen is a trigger for starting up the alkaloid production, rather than the limitation of carbon. Nitrate limitation influenced several enzyme activities in primary metabolism, e.g. phosphofructokinase and pyruvate kinase, while cell viability remained at a high level for several days [83].

Addition of organic nitrogen, e.g. casein, resulted in decreased yields of tryptamine [85].

3.1.3 Phosphate

The fact that phosphate plays an important role in the regulation of growth and secondary metabolism and that it is rapidly and completely taken up from the medium, were motives for many detailed investigations on phosphate metabolism. The rapid uptake of phosphate is a commonly observed phenomenon [78, 84]. Phosphate is usually completely taken up within the first day(s) of the growth cycle. Ashihara et al. [86, 87, 88] described the metabolic fate of phosphate; after a rapid uptake most of the inorganic phosphate (P_i) is stored in the vacuole. With the growth, the amount of free P_i is decreasing and in the stationary phase 85% of the added phosphate has been built into organic compounds, 45% into nucleinic acids, 30% into phospholipids, 5–10% into nucleotides and less than 3% into sugar phosphates and proteins. The rapid uptake of phosphate leads to a strong increase in ATP synthesis during the lag phase of the cell growth cycle, which seems to be essential for subsequent cell growth. It was shown that phosphate could be taken up against a gradient of three orders of magnitude [89]. Knobloch et al. [89, 90] determined the influence of initial phosphate concentration on product formation (alkaloids and phenolics) and related enzyme activities (tryptophan decarboxylase (TDC) and phenylalanine ammonia-lyase (PAL), respectively). TDC activity was not influenced by the initial phosphate concentrations in the induction medium. At a concentration as low as 0.05 mM, PAL activity was reduced to 24% of a control (standard MS medium contains 1.2 mM phosphate). An increase in initial phosphate stimulated growth and protein synthesis. In spite of TDC activity, high levels of phosphate inhibit alkaloid formation, probably caused by a shortage of tryptophan. Production of phenolics is also inhibited by phosphate; low initial phosphate concentration cause a strong inhibition of PAL activity, high initial concentrations of phosphate inhibited PAL and caused also a shortage of phenylalanine. The inhibitory effect of phosphate on alkaloid production was previously observed [39, 41, 49]; the induction [39] and production [41] media for alkaloids are based on low concentrations or complete absence of phosphate.

^{31}P-NMR proved to be a powerful tool for studying phosphate metabolism in cultured cells [91]. Phosphate is stored in the vacuole (pH < 5.7), from which a constant cytoplasmic concentration (pH $= 7.3$) is obtained. Due to their different spectral shifts, various phosphor-containing metabolites can be observed in the spectra, e.g. sugar phosphates, ADP, ATP and nucleoside diphosphoglucose.

3.1.4 Minerals and vitamins

MacCarthy et al. [92] studied the influence of the removal of calcium,

magnesium, potassium, sodium and sulphate from a modified Wood & Braun medium [93]. None of these cations was completely removed during culture growth. Uptake of sodium and potassium from the medium proceeded in parallel with culture growth. Residual magnesium, sulphate and calcium concentrations in the medium fall during growth phase. During the later part of the culture period, increases were observed for sulphate, sodium and potassium, which was associated with loss of cell viability. The level of calcium continued to fall throughout the culture period probably due to the binding of this ion to polymolecular material resulting from cell lysis. Knobloch & Berlin [39, 81] investigated the influence of magnesium and calcium salts (added to an induction medium; 8% sucrose) on the production of secondary metabolites. These ions did not influence alkaloid and polyphenol accumulation. Addition of all other micronutrients and vitamins of the MS medium to the sucrose induction medium even so did not suppress alkaloid accumulation. Recently, ten Hoopen et al. [84] studied the uptake kinetics of all LS medium [94] components (except for the growth regulators) in order to obtain some insight in the cause of a pH dip in the medium during the lag phase observed after inoculation. Of the micronutrients, only the cations Zn and Mo were taken up completely, while SO_4, Ca, K, Mg, Mn, Na and Fe were taken up to maximally 50% of the initial amount. The pH dip seemed not to be caused by an exchange of protons for cations, but probably by a release of acid vacuole contents during cell shrinkage.

Removal of the vitamin supplement (thiamine-HCl, pyridoxine, nicotinic acid and inositol) did not influence culture growth substantially over a period of 2 years. Alkaloid production, however, was reduced and qualitatively changed [80].

3.1.5 Phytohormones, prototrophy

2,4-dichlorophenoxy acetic acid (2,4-D) plays an inhibitory role on alkaloid and anthocyanin accumulation [39, 41, 65, 95]. The intracellular 2,4-D concentration regulates alkaloid formation [96, 97], while anthocyanin accumulation is decreased by a decreased number of producing cells [95]. Addition of naphtaleneacetic acid (NAA) and gibberellin (GA_3) also suppressed alkaloid formation, while addition of indoleacetic acid (IAA) resulted in both the highest biomass and alkaloid yields. Addition of benzylaminopurine (BAP) gave highest alkaloid yields, therefore a mixture of 10^{-6} M IAA and 5×10^{-6} M BAP is used in the alkaloid production medium [41].

Merillon et al. [98, 99] observed an increased ajmalicine and serpentine production after one passage without 2,4-D. Prototrophic or habituated cell lines, which were subcultured on media without 2,4-D and kinetin, accumulated significantly higher amounts of alkaloid, which arose neither from

a higher level in tryptophan nor from an increase in tryptophan decarboxylase (TDC) and strictosidine synthase (SSS) activities. The habituated cell lines were not stable with respect to the alkaloid production, but after one year of subculturing, product yields were still higher than in the original non-habituated strains.

Habituated cell lines respond less to environmental conditions than non-habituated cell lines, e.g. the use of production media to increase alkaloid production [66, 99]. Eilert et al. [66] compared alkaloid production by a habituated cell line with that of a tumorous cell line. Addition of 2,4-D to these cell lines decreased alkaloid production and TDC and SSS activities were inhibited. Elicitation with a *Pythium* homogenate or induction with media did not influence alkaloid accumulation in these culture types.

Palni [100] studied cytokinin accumulation in the culture medium of crown-gall tissue. One week after subculturing, cytokinin activity was detectable in the solidified medium. Maximal activity was observed after 5–6 weeks; zeatin and several metabolites were identified.

Addition of abscisic acid (ABA) to suspension cultures of *C. roseus* stimulated intracellular accumulation of catharanthine and ajmalicine [101]. ABA had no significant effects on growth. ABA addition to cells in a 30 l fermenter resulted in a catharanthine yield of 85.25 $mg\,l^{-1}$ after ten days of cultivation.

3.2 Other factors influencing secondary metabolism

3.2.1 Light, autotrophy

The fact that the dimeric indole alkaloids vinblastine and vincristine can be isolated from leaves but not from heterotrophic suspension cultures initiated studies on photoautotrophic suspension cultures. Sustained photoautotrophic growth was obtained in a sugar-free B5 medium containing naphtaleneacetic acid and kinetin in a CO_2-enriched atmosphere. However, photoautotrophic cell suspension cultures did not accumulate vindoline or dimeric indole alkaloids [63]. Aspects of photoautotrophic cell cultures have been reviewed [102]. Irradiation of heterotrophic suspension cultures with fluorescent light resulted in increased accumulation of serpentine, phenolics and anthocyanins [49]. The accumulation of serpentine was preceded by an increase and subsequent decrease of its biogenetic precursor ajmalicine, which was the predominant alkaloid of the culture on induction medium in the dark. Knobloch et al. [49] observed that effects of light were largely dependent on the medium composition, i.e. the optimum effect of light to stimulate the formation of anthocyanins and serpentine required low concentrations of 2,4-D, phosphate and mineral nitrogen.

With respect to anthocyanin production, the irradiance is linear to the number of pigmented cells, anthocyanin yield and anthocyanin concentration [95, 103].

In callus cultures the light-dependent formation of serpentine and anthocyanins [104, 105] have been reported. From callus cultures with a high chlorophyll content, increased concentrations of dimeric indole alkaloids could be isolated [106].

Light plays an important role in the developmental regulation of vindoline biosynthesis [107] (see Chapter 4).

Various aspects of light on growth and production in plant cell cultures have been reviewed [108].

3.2.2 Temperature

By lowering the temperature from 27 °C to 16 °C, Courtois & Guern [109] observed a strong increase in alkaloid content. However, due to the strongly reduced growth rate at this temperature, the alkaloid yield per litre medium is not changed substantially. At 16 °C the vacuoles of the cells were increased in size and the increased alkaloid content resulted probably from a modified balance between synthesis and degradation. The alkaloid content was strongly reduced by increasing the temperature to 38 °C. Doubling times of cell populations and total cell cycling times were unaffected between 20 °C and 27 °C.

These findings have been verified by Morris [110, 111]. He observed optimum growth at 35 °C with doubling times of 20 h. Optimum serpentine production was obtained between 20 °C and 25 °C, while ajmalicine was produced at optimum at 25 °C. Both for biomass and alkaloid production the curves of yield vs temperature showed sharp maxima. By changing the temperature, the serpentine/ajmalicine ratio can be influenced, however, care should be taken for the fact that maximal yields of these alkaloids are obtained at different stages of growth. Furthermore, some seasonal variation might occur, caused by slight temperature differences in the climate chamber in summer and winter season [111].

Lowering the temperature from 25 °C to 15 °C influences also the fatty-acid composition of suspension-cultured cells: the formation of oleic and linolenic acid was enhanced [112]. However, this is a rather general phenomenon and it is connected to the preservation of optimum membrane fluidity [113].

3.2.3 pH of culture media

The time course of the pH of the culture medium from batch-cultured cells shows after an initial pH dip a slow increase to more neutral values. The initial dip is probably caused by a release of acid vacuole contents [84].

The initial pH value of the alkaloid production medium adjusted to pH 5.5, 6.0 or 6.5 did not influence alkaloid production significantly [27]. Döller [85] found a pH of 5.5 to be the optimum for the production of serpentine. One particular cell line (PRL 953) accumulated more alkaloids when grown in production medium with pH 7.0 than with pH 5.5 [114].

3.2.4 Aeration

Oxygen supply of suspension-cultured cells in shake flasks is, amongst other things, determined by the permeability of the plug or stopper of the flasks and the intensity of shaking. The oxygen transfer through several stoppers has been compared recently. A cotton wool plug is highly permeable for oxygen but the reproducibility of this type of stopper is low. Closing the flasks by two layers of aluminium foil resulted rapidly in an oxygen-limited growth of the cultures. However, silicon foam stoppers combine a good permeability with a good reproducibility [115]. With this type of stopper a growth-curve of a suspension culture in a single culture flask can be determined by measuring the loss of weight caused by dissimilation [116].

The oxygen supply in fermentors depends strongly on the type of fermentor. The use of airlift fermentors was preferred because of the high oxygen transfer rate combined with low shear stress for the plant cells. However, recently more experimental data came available on growth in stirred fermentors. In such fermentors, cultured plant cells showed little sensitivity for shear stress [115]. Pareilleux & Vinas [117], Ducos & Pareilleux [118] and Hegarty et al. [119] studied the effect of the aeration rate on culture growth. Too high and too low rates lead to a suboptimum in dissolved CO_2 concentrations, which inhibited growth. Maurel & Pareilleux [120, 121] observed a growth-promoting effect of carbon dioxide; the rate of fixation by the heterotrophic cultures depended on the partial pressure.

Mass cultivation of *C. roseus* cells in fermentors has been described [122, 123]. Bailey et al. [124] described a model of growth, nutrient uptake and product formation. Aspects of alkaloid production during upscaling up to 5000 l fermentors has been studied recently by Schiel & Berlin [125]. Bioreactor design and configuration with respect to production of metabolites by plant cell cultures has been discussed by Shuler & Hallsby [126].

3.2.5 Osmotic stress

By exposure of suspension-cultured plant cells to hyper-osmotic solutions, release of cell contents can be obtained and the size of the cells can be influenced. These aspects are of importance for cryopreservation procedures, mass cultivation, immobilization and permeabilization. Osmotic stress, obtained by the addition of mannitol to culture media, inhibited

cell division, increased cell viability and increased cell alkaloid production [127]. The release of alkaloids and enzymes from suspension-cultured cells into media with a high ionic strength have been reported [128].

3.3 Additives to culture media with the aim to increase product yield

Several organic compounds, pure or as complex mixtures, such as extracts of micro-organisms, have been added to culture media, in general with the aim to increase product yield. The addition of several compounds was based on knowledge of biosynthetical pathways in order to increase the availability of alkaloid precursors. This can be reached by simply adding the precursors or by interfering with the precursor metabolism. The latter possibility is usually based on a selection of certain cell lines, e.g. 5-methyltryptophan-resistant cell lines. These selections have been discussed in Chapter 2.

Extracts of micro-organisms or, in more general terms, biotic elicitors, have been added to cultures with the aim to obtain a possibility to induce alkaloid biosynthesis selectively.

3.3.1 Addition of precursors

Table 5 summarizes the effects of additives that were added to suspension cultures with the aim to increase the availability of alkaloid precursors. Other compounds were added to influence alkaloid metabolism more indirectly, e.g. by influencing cell differentiation and growth. The additives named in Table 5 are classified by the site of action on alkaloid metabolism.

Increased alkaloid production was obtained by feeding with l-tryptophan, tryptamine, secologanin, loganin, loganic acid and shikimic acid. Stafford & Fowler [71] fed tryptophan and secologanin to 5-methyltryptophan-resistant cell lines and to non-resistant cell lines. In all cases, the adition of secologanin alone resulted in a reduction in the intracellular tryptophan level and an increase in the level of serpentine. In a resistant cell line a 37-fold increase was observed. The addition of tryptophan alone to the resistant cell lines resulted in almost complete suppression of serpentine accumulation. Addition of both tryptophan and secologanin did not result in a higher serpentine accumulation than obtained by feeding with secologanin alone. Induction of the terpenoid pathway by organic amines increased alkaloid accumulation [131, 132]. These results indicate a possible limitation on alkaloid yield at the point of secologanin synthesis.

3.3.2 Elicitation

Treatment of suspension cultures with elicitors offers possibilities to shorten the production time. The product is often excreted into the medium and thus

Table 5. Additives to culture media aimed to increase alkaloid production by various mechanisms.

Additive	Result/product formed[a]	Ref.
a) By addition of alkaloid precursors		
l-tryptophan	alkaloid +	41
d-tryptophan	alkaloid −	41
tryptamine	N-acetyltryptamine	
	N,N-dimethyltryptamine	
	alkaloid +	129
	alkaloid −	41
shikimic acid	alkaloid +	41
quinic acid	alkaloid −	41
anthranilic acid	alkaloid −	41
indole	alkaloid 0	41
geraniol	alkaloid 0	129
	alkaloid −	41, 85
loganin	alkaloid +	41
loganic acid	alkaloid +	41
secologanin	alkaloid +	41, 130
	alkaloid 0	129
	intracellular tryptophan −	
	serpentine +	71
secologanic acid	alkaloid −	41
mevalonic acid lactone	alkaloid 0	129
b) By induction of terpenoid biosynthesis by derepression		
1,1 dimethyl-piperidine	alkaloid +	131, 132
2-diethylamino-2,4-dichlorophenylether	alkaloid +	131
2-diethylamino-ethyl-β-naphtylether	alkaloid +	131
2-diethylamino-ethyl-3,4-dimethyl-phenylether	alkaloid +	132
c) By induction of cell differentiation via inhibition of DNA methylation		
5-azacytidine	lirioresinol B mono-β-glucoside	62
d) By induction of cytochrome P-450		
butylated hydroxytoluene (BHT)	dimerisation to substituted stilbene quinone	133
e) Addition of co-factors		
SAM	vindoline 0	114
l-methionine	alkaloid −	41
f) By influencing culture growth via IAA metabolism		
ferulic acid	alkaloid +	134
caffeic acid	alkaloid +	134

[a] +: increased production; −: decreased production; 0: no influence on production.

the cells can be recycled and re-elicited. Factors determining product yields are elicitor specifity, elicitor concentration, duration of exposure, cell line specifity, composition of culture medium and timing of elicitor addition [135]. Eliciting secondary metabolism in plant cell cultures has been reviewed [136, 137].

Elicitation of a suspension culture with *Pythium aphanidermatum, Alternaria zinnae, Verticillium dahliae* or *Rhodotorula rubra* resulted in an accumulation of strictosidine lactam, ajmalicine, lochnericine, catharanthine and tabersonine. Maximal product yield was obtained with a *Pythium* homogenate added to a 10-day-old suspension culture. The moment of addition determined the products formed: addition to a 2-day-old culture yielded tryptamine, to a 5-day-old culture N-acetyltryptamine, to a 10-day-old culture alkaloids, and addition after 15 days caused a browning of the culture. About 40% of ajmalicine could be recovered from the medium; accumulation of alkaloid did not depend on production media [138]. The alkaloid accumulation was preceded by a rapid transient increase in the tryptophan decarboxylase (TDC) and strictosidine synthase (SSS) activities caused by a de novo enzyme synthesis. In cells cultured on alkaloid production medium and also after elicitation, SSS is induced many hours before TDC, suggesting that TDC is regulated by intracellular tryptamine concentrations [139]. The *Pythium* elicitor preparation did not influence alkaloid production in tumourous and habituated cell lines (see Section 3.1.5 [66]).

It must be emphasized that only a few selected cell lines react on elicitation with an increased alkaloid production. Eilert et al. [138] distinguished three types of cell lines:

1. Cell lines which do not respond to elicitation or production media.
2. Cell lines which react with brown colouration of medium and cells, an unspecific reaction to stress.
3. Cell lines which react with browning and increased alkaloid production.

Cultures of *C. roseus* exposed to crude culture filtrates or proteinaceous extracts from cultures of the fungi *Pythium aphanidermatum, Eurotium rubrum, Micromucor isabellina* and *Chrysosporium palmorum* accumulated increased amounts of tryptamine, ajmalicine and catharanthine as well as dark pigments [140, 141]. Addition of vanadyl sulphate (10–500 $mg\,l^{-1}$) to suspension cultures of *C. roseus* increased intracellular accumulation of ajmalicine and catharanthine [142]. The response depended upon cell line, concentration of vanadyl sulphate and the stage of the growth phase at which the cells were treated and was also demonstrated in fermenter systems (30 l).

It is not likely that the alkaloids act as phytoalexins. Treatment of suspension cultures of *Tabernaemontana* species, which also belong to the

family Apocynaceae, with several biotic elicitors resulted in a rapid de novo synthesis of triterpenes of the ursene carboxylic acid type [143]. Such triterpenes could not be isolated from an elicited *C. roseus* suspension culture. However, treatment with cellulase or pectinase of this culture resulted in a strongly blue colouring of the cells and the media, probably due to anthocyanins, whereas *Candida albicans* treated cells only showed a yellow colouration.

To our knowledge no further information on the phytoalexins in *Catharanthus* is available.

4 Regulation of secondary metabolism

Product accumulation is a result of the regulation of secondary metabolism. It includes product formation, storage, excretion, uptake and degradation in each individual cell. Since alkaloid production is thought to be heavily regulated, these compounds are probably of great physiological importance for the plant.

Although no reports exist on the allelochemical properties of the *C. roseus* alkaloids, activities against herbivores and insects in general may be assumed. The biological activities of the alkaloids [4] support this assumption. Frischknecht et al. [144] studied the influence of drought and wounding stress on alkaloid accumulation in *C. roseus* plants. In healthy, non-stressed plants the very youngest part of the shoot tip contained more than 3% alkaloid; from top to base alkaloid content of consecutive leaf pairs decreased. Under drought stress, the concentration of alkaloid within the plant differed little, still supplying the same effectiveness in chemical defence. Wounding stress increased alkaloid content up to 100%. In non-growing tissue, alkaloid accumulation was not enhanced by wounding.

Mersey & Cutler [145] studied the distribution of catharanthine and vindoline in leaves of *C. roseus*. Iodoblasts, large, refractile and autofluorescent cells in the mesophyll, are enriched in these alkaloids. Such cellular localization might have significance for designing production systems of these alkaloids by tissue cultures.

The plant-developmental regulation of the biosynthetic pathway from tabersonine to vindoline (Fig. 3) has been described by De Luca et al. [107]. Growth of seedlings in the dark resulted in an early ubiquitous accumulation of tabersonine as a major alkaloid, and in the subsequent 5–10 days, accumulation of vindoline and its immediate precursors was restricted to the cotyledons. Light enhanced vindoline biosynthesis, although it was not essential. It was suggested that the tabersonine biosynthetic pathway

enzymes occur in all plant parts, whereas the last 5 steps in vindoline biosynthesis are restricted to the aerial parts of the plant and that the whole pathway to vindoline is developmentally regulated. The majority of genes controlling indole alkaloid biosynthesis must be expressed in early germination [107].

Chapter 2 and 3 summarized various factors influencing secondary metabolism in cell and tissue cultures of *C. roseus*. Product accumulation proved to be dependent on the degree of differentiation of the cultured cells and of various environmental conditions. Optimization of these variables resulted in high product accumulation (see e.g. [41, 53, 65]). In suspension-cultured cells, alkaloid accumulation occurred during the whole culture cycle except for the period of active mitosis, where alkaloid biosynthesis was reduced. After the peak of mitotic activity but before carbon, nitrogen or phosphate limited cell growth, alkaloid production resumed [27, 65]. Other factors influencing product yields are: age of inoculum [111, 146], inoculum density [65, 111] and culture (in)stability [147]. It should be emphasized here again that the variability observed in growth rates and alkaloid yields might be due to the inadequate control of subculture methodologies and environmental conditions [111].

Stafford et al. [146] found that none of the production media tested contained more than 50% alkaloid-accumulating cells. Relatively undifferentiated and fast growing cells are 'plastic' and differentiate only after a right stimulus. Such a stimulus might for example be obtained by the use of biotic elicitors. Elicitation and the use of production media resulted in similar product yields, which might be produced by the same amount of 'plastic cells'.

The regulation of anthocyanin production was studied by Hall & Yeoman [95, 103, 148]. By using a microdensitometer, the contribution of each individual cell to the total product yield could be determined. The intracellular anthocyanin concentration varies very little and is therefore likely to be controlled by a feedback inhibition mechanism. The proportion of cells accumulating anthocyanins, however, varied considerably in cultures grown under different conditions, but nevertheless remained low, never exceeding 20% of the total cell population. Increased concentrations of 2,4-D decreased the number of producing cells and thus the total anthocyanin production decreased. Depletion of phosphate and nitrogen increased product yield through an increased percentage of pigmented cells.

In suspension-cultured cells, alkaloids are stored inside the vacuole. Enzyme involved in the biosynthesis of the alkaloids, as strictosidine synthase and a specific glucosidase were found to be located in the cytosol [149]. Deus-Neumann & Zenk [149] found the transport of alkaloids across

the tonoplast to be an active, energy-requiring mechanism, sensitive to pH and temperature. Serpentine, however, was not taken up by isolated vacuoles; nevertheless it is stored in the vacuole. It was presumed that not serpentine itself but the precursor ajmalicine is taken up by the vacuoles. The uptake of alkaloids across the tonoplast proved to be highly specific, which means that the transport system only recognizes alkaloids indigenous to the plant from which the vacuoles have been isolated. They also found that vindoline was not trapped inside the vacuole as a non-diffusable alkaloid salt. Therefore, the ion-trap mechanism was excluded as an explanation for alkaloid transport into the vacuoles. This ion-trap mechanism (proposed by a.o. [150–152]) was based on the observation that 'alkaloid-storage cells' exhibited a vacuolar pH of 3, whereas culture medium and 'normal' cells of a suspension culture had a vacuolar pH of about 5. Alkaloid accumulation is driven by this difference in pH; the alkaloids are taken up in their unprotonated forms, trapped and accumulated inside the vacuole by protonation.

As the amount of alkaloid stored in the vacuole determines the productivity of the cell culture (if no excretion of product into the medium occurs), the studies on the mechanisms of compartmentation and transportation are extremely valuable.

Product accumulation is the balance of product formation and product degradation. Most papers dealing with increased product yields emphasize on the regulation of product synthesis. Knowledge on alkaloid catabolism is lacking, only the active degradation of vindoline and catharanthine has so far been reported. Morris [153] observed a rapid metabolism of vindoline and catharanthine in leaf tissue during initiation of callus. From this callusing tissue, serpentine and ajmalicine could be isolated. Radio-labelled vindoline and catharanthine were extensively degraded to non-alkaloidal material [114]. Turnover and degradation of secondary products has been reviewed by Barz & Köster [154].

5 Biosynthesis, biotransformation and enzymology

On the biosynthesis of the more than 90 alkaloids isolated from *C. roseus* some insight is obtained by feeding experiments with labelled precursors [11, 156]. The chemical synthesis of the important vinblastine group, which is based on the biomimetic modified Polonovski reaction, has been reviewed by Potier [157].

Interference in the secondary metabolism, e.g. by manipulating the enzy-

Table 6. Characteristics of enzymes involved in the biosynthesis of indole alkaloids in *C. roseus* [after 158].

Enzyme	Co-enzyme[a]	Isol. from[b]	MW kd	K_m value	Specific	pH opt.	Ref.
monoterpene hydroxylase	NADPH	p		5.5 μM geraniol 11 μM nerol	+		172
SAM: loganic acid methyl-transferase	SAM	p		12.5 mm loganic acid	+		173
tryptophan decarboxylase	PP	s	115	75 μM l-tryptophan	+		174
strictosidine synthase		s	38	0.83 mM tryptamine 0.46 mM secologanin	+		164
		s	34	2.3 mM tryptamine 3.4 mM secologanin	+	4–8 6.8	163
glucosidase I		s	230	0.2 mM strictosidine	+	6–6.4	168
glucosidase II		s	> 450	0.1 mM strictosidine	+	6–6.4	168
glucosidase A		p	182		–		169
glucosidase B		p	120		–		169
glucosidase C		p	55		–		169
glucosidase D		p	8		–		169
glucosidase C		s	400		–		169
glucosidase GII		s	55		+		169
geissoschizine dehydrogenase	$NADP^+$	s		83 μM geissoschizine	+	7.6	170

tetrahydro-alstonine synthase	NADPH	s	81	62 μM cathenanamine	+	6.6	171
acetyl-CoA: 17-O-deacetyl-vindoline 17-O-acetyl transferase	acetyl-CoA	p			+		175 176
SAM: 11-O-demethyl-17-O-deacetyl-vindoline 11-O-methyl transferase	SAM	p			+		177
acetylesterase		p,s			−		177

[a] PP: pyridoxal phosphate; SAM: S-adenosyl-L-methionine.
[b] p: plant; s: suspension culture.

mes or the expression of the related genes, requires fundamental knowledge of the enzymes involved in the biosynthesis of the indole alkaloids [158].

The building stones of the indole alkaloids, tryptamine and secologanin, in their turn need precursors from the primary metabolism. The enzymes which perform their activity on the border between primary and secondary metabolism are thought to have a regulatory function in secondary metabolite formation.

Tryptamine is available for secondary metabolism by the activity of tryptophan decarboxylase, an enzyme which catalyses the conversion of tryptophan to tryptamine. The activity of this enzyme can be induced by media containing high concentrations of sucrose [90]. Its regulatory function has been confirmed by several experiments, its biological half-life was estimated to be 21 h [159]. Some assays for measuring the activity have been described. Merillon et al. [130] compared indole alkaloid accumulation and TDC activity of a *C. roseus* suspension culture on three different media. They did not observe a correlation between maximum enzyme activity and alkaloid content, suggesting the terpenoid pathway to be the limiting factor in alkaloid production.

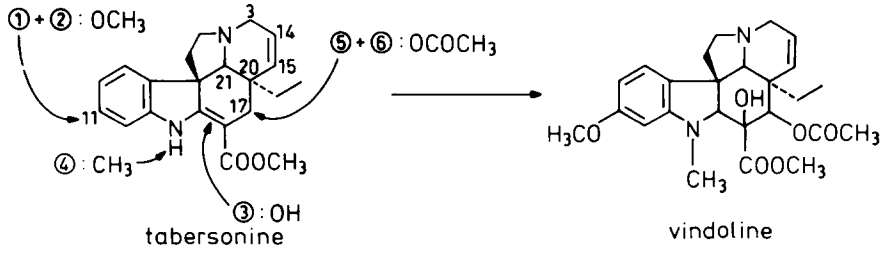
On the biosynthesis of secologanin, substantial experience has recently been gained. The intermediacy of geraniol, 10-oxogeranial/10-oxo-neral and iridodial was demonstrated [160]. The enzyme geraniol-10-hydroxylase can be induced by production media and it is strongly enhanced just before product accumulation, which might point towards a regulatory function of this enzyme in the pathway to secologanin [161, 162]. In the experiments an intermediate accumulation of tryptamine was observed, which was later incorporated into ajmalicine; indicating a non-synchronized coordination of the two precursor pathways.

Tryptamine and secologanin are stereospecifically condensed by strictosidine synthase. This enzyme yields the 3α-isomer. After isolation and characterization of the enzyme [163, 164], Pfitzner & Zenk [165] were able to immobilize the enzyme, which enabled them to produce relatively large amounts of strictosidine. Recently, Pennings et al. [166, 167] developed HPLC assays for tryptophan decarboxylase and strictosidine synthase, which have the advantage that no labelled precursors are needed.

After the condensation the biosynthesis proceeds by elimination of the glucose residue. Specific [168] and non-specific [169] glucosidases have been characterized. Within the resulting corynanthean type of alkaloids two enzymes have been partially characterized, namely geissoschizine dehydrogenase [170] and tetrahydroalstonine synthase [171].

Some characteristics of the various enzymes are presented in Table 6.

The inability of suspension cultures of *C. roseus* to produce the dimeric



	Enzyme	Product
1.	hydroxylase	11-hydroxytabersonine
2.	methyltransferase	11-methoxytabersonine
3.	hydroxylase	11-methoxy-2,16-dihydro-16-hydroxytabersonine
4.	methyltransferase	N(1)-methyl-11-methoxy-2,16-dihydro-16-hydroxytabersonine (desacetoxyvindoline)
5.	hydroxylase	desacetylvindoline
6.	acetyltransferase	vindoline

Fig. 3. Proposed biosynthetic pathway from tabersonine to vindoline in *C. roseus* seedlings [after 107].

indole alkaloids vinblastine and vincristine is a result of the incapability of the cultures to produce the dimeric moiety vindoline. The late steps of vindoline biosynthesis have been studied in detail [106, 175–177] and the possible pathway is presented in Fig. 3.

The expression of the late steps is developmentally regulated and vindoline production is related to aerial parts of the plant. The latest step in the biosynthesis is acetyl-CoA dependent, which makes a cell-free synthesis of vindoline [178] from tryptamine and secologanin unlikely [179].

The enzymes involved in the dimerization process have been the subject of several investigations.

A cell-free conversion of 3′,4′-anhydrovinblastine to vinblastine (Fig. 4) was demonstrated [178, 180–184]. Endo et al. [185] reported a cell-free production of 3′,4′-anhydrovinblastine from vindoline and catharanthine with a maximum yield of 22% from the substrate. Other dimeric alkaloids, leurosine, catharine, vinamidine and 3(R)-hydroxy-vinamidine were also found [185, 186]. Table 7 summarizes biotransformation studies of alkaloids and precursors. Growing cells of a non-producing cell line and peroxidase can both convert 3′,4′-anhydrovinblastine to leurosine and catharine. In 'spent medium', significant enzyme activity could be detected. Enzyme purification, biosynthesis and biotransformation of 3′,4′-anhydrovinblastine has been reviewed [182, 186].

Biotransformation and the site of action of 14 growth-inhibitory trypto-

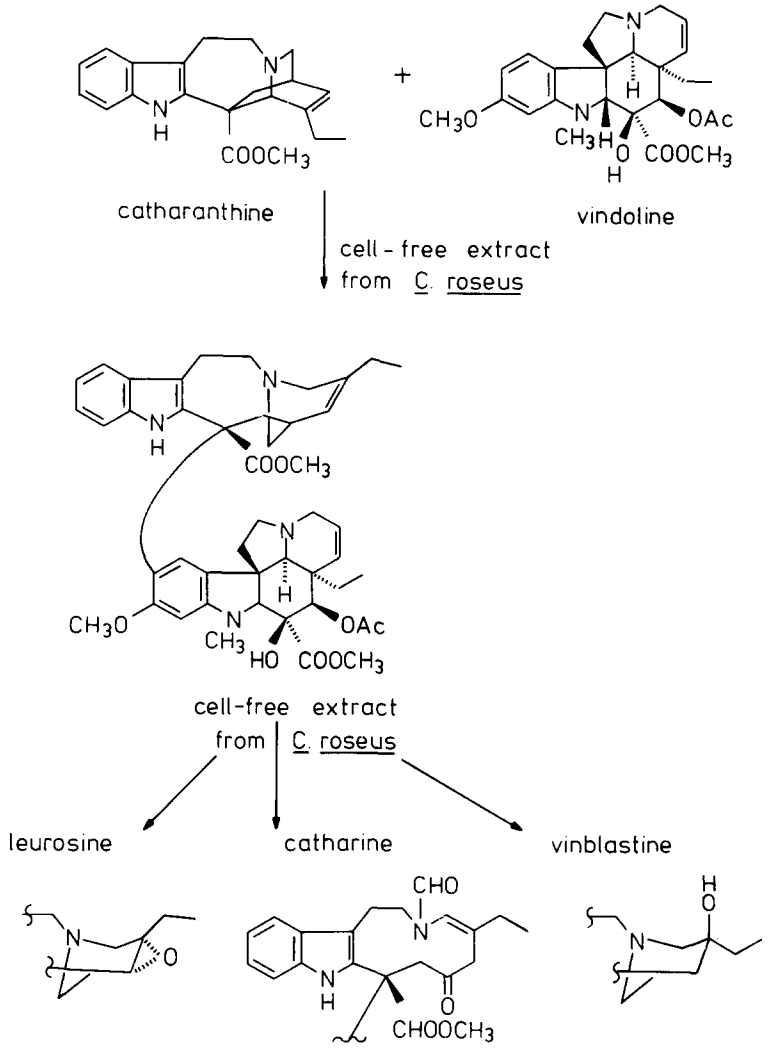


Fig. 4. Cell-free biotransformation of *C. roseus* alkaloids [158].

phan analogues has been studied [189]. Only 4 of the analogues (4-methyl-, 4-fluoro-, 5-fluoro-, and 5-hydroxytryptophan) were good substrates for TDC, while α-methyltryptophan was a competitive inhibitor of this enzyme. None of the analogues was efficiently incorporated into protein. The toxicity of 12 of the analogues was directly correlated with their inhibitory effect on anthranilate synthetase activity.

The *Catharanthus* alkaloids have also been subjected to microbial and enzymatical transformations [190–192].

Table 7. Biotransformation of precursors and/or alkaloids by enzymes from *Catharanthus roseus* (see also Table 5).

Alkaloid/ precursor	Source[a]	Product	Ref.
10-hydroxy-geraniol	s	10-hydroxylinalool 10-hydroxycitronellol 7,8-dihydro-10-hydroxygeraniol dihydrocitronellol 7,8-dihydro-10-hydroxylinalool	187
8-hydroxy-7,8-dihydrogeraniol	s	(−)-8-hydroxy-7,8-dihydro-citronellol	187
(+)-10-hydroxy-citronellol	s	mixture of diastereomers	187
vindoline	s	desacetylvindoline	43
	s	dihydrovindoline	188
vindoline + catharanthine	cf	3′,4′-anhydrovinblastine	169, 183, 185, 186
	cf	leurosine	169, 183, 185, 186
	cf	catharine	185, 186
	cf	vinamidine	185, 186
	cf	hydroxyvinamidine	185, 186
3′,4′-anhydro-vinblastine	l,cf,gc	vinblastine	178, 183–185
	cf,gc,sm,p	catharine	185
	cf,gc,sm,p	leurosine	185
	gc	vincristine	185
	gc	4′-deoxyleurosidine	185
	gc	vinamidine	185
	gc	hydroxyvinamidine	185
vinblastine	cf	vinamidine	185
	cf	N-formylvinamidine	185
vincristine	cf	N-formylvinamidine	185

[a] l: leaf; cf: cell-free; gc: growing cells; sm: spent medium; p: peroxidase; s: suspension culture.

Most enzymes are very specific. However, peroxidases, alcoholdehydrogenases, methyltransferases and glucosidases are known to be relatively unspecific and these enzymes can be used for the transformation of xenobiotics. Table 8 presents some of the biotransformations of xenobiotics by enzymes of *Catharanthus*.

Detoxification of heavy metals like cadmium is obtained by complexation to metalothioneins [194] and/or phytochelatins [195]. The phytochelatins isolated by Grill et al. [195] have the following structure (γ-glutamic acid-cysteine)$_n$-glycine (n = 3–7) and can be regarded as secondary metabolites.

Table 8. Biotransformation of xenobiotic compounds by cell cultures of *Catharanthus roseus*.

Substrate	Product	Ref.
aniline	3-hydroxyaniline acetanilide	193
benzoic acid	4-hydroxybenzoic acid	193
anisole	phenol	193
coumarin	7-hydroxy-coumarin	193
BHT	substituted stilbene quinone	133
Cd	Cd-thionlike proteins	194
	phytochelatin	195
progesterone	Δ^4-pregnen-20β-ol-3-one	196, 197
	Δ^4-pregnen-14α-ol-3,20-dione	196, 197
cyclohex-2-en-l-one	cyclohexanone	198

6 Analysis of alkaloid production

For the isolation of alkaloids from plant material the characteristic property of alkaloids, namely its alkaline character, is often used. This allows an extraction of the alkaloids at alkaline pH with organic solvents such as chloroform and ethyl acetate. A crude extract can be freed from acids, lipids, phenolics etc. by an extraction at acidic pH. The crude extracts are usually obtained by extracting biomass with (m)ethanol or ethyl acetate. This 'classical' method is still used by various groups in many modifications. Hofmann et al. [45] improved the extraction method of vinblastine from leaves and tissue cultures and were able to detect a content of 0.00013% in a callus culture using reflectance densitometry. The disadvantages of the classical method are that it is usually time consuming, less reproducible for small sample sizes and it demands large volumes of organic solvents. These disadvantages can be greatly overcome by using a solid phase extraction (SPE) method. The SPE is particularly being used in clinical chemistry where small amounts of drugs often have to be measured in large volumes of body fluids. These drugs are then concentrated on a cartridge (or preconcentration column) containing silica or silica with chemically bound C18, C8 or ion-exchange groups. Nowadays, SPE is also the method of choice for the determination of alkaloids in plant cell cultures.

Kohl et al. [199] compared the classical extraction method, based on liquid-liquid partitioning with two SPE methods. One method used kieselgur as solid phase, the second one was based on ion-exchange chromatography. It was concluded that SPE, in particular when using kieselgur, allows a quicker and more effective sample clean up as the classical method.

Table 9. Solid-phase extraction methods for the extraction of indole alkaloids from cell and tissue cultures of *C. roseus*.

Solid phase	Type of extract concentrated on solid phase	Sample clean up	Elution of alkaloids	Ref.
Kiesel gur	methanolic extract + 10% acetic acid	hexane 6 ml	1. NH_3/air 2. chloroform 6 ml	199
Kation-exchange	methanolic	methanol 0.5 ml	2% triethanol-amine in methanol 2 ml	199
C18	0.01% acetic acid pH 4.0 (set to pH 7.3–7.5 before concentration)	methanol: 5 mM diammonium-hydrogen-phosphate pH 7.3 10:90 60 ml	methanol: 25 mM diammonium-hydrogen-phosphate pH 4.7 85:15 2 ml	200
C18	methanolic extract: 0.05 M n-HS[a] 1:3[b]	—	methanol: 0.05 M n-HS[a] 95:5 4 ml	65

[a] n-heptanesulphonate.

[b] Before concentration the column was activated with 10 ml acetonitrile, 10 ml 95% methanol containing 0.05 M n-HS and 10 ml aqueous nHS, respectively.

Since this report of Kohl, also some other SPE methods have been reported (Table 9).

Extraction was performed by using straight phase (kieselgur), reversed phase (C18) or ion-exchange solid phases. Already by these few reports the versatility of SPE was demonstrated. Vendrig et al. [201] however, reported, variable recoveries of dimeric indole alkaloids (vinblastine and vincristine) due to non-reproducible diol-column packings.

The extracts obtained by SPE can be used directly or after concentration for further analysis: i.e. HPLC. In Table 10, some HPLC systems are described which are used for analysis of extracts obtained by SPE or by classical extraction. All HPLC systems used were based on reversed phase chromatography using C18 as stationary phase, however, large differences in selectivity of some reversed phase stationary phases were observed [199].

The analytical system, i.e. extraction and HPLC analysis, should be

Table 10. HPLC systems for the analysis of alkaloid extracts.

Extraction method[a]	Stationary phase	Mobile phase[b]	Detection	Ref.
Solv. part.	C18	H_2O:CH_3CN 62:38 + 0.1% Et_3N	UV 254 and 280 nm	25
SPE	C18	Gradient system 1: (A) MeOH:H_2O:TEA 40:60:0.5 (B) MeOH:TEA 100:0.5 Gradient system 2: (A) CH_3CN:0.01 M TEA-formate buffer, pH 8.5 20:80 (B) id. 80:20	UV	199
SPE	C18	MeOH:5 mM diammonium hydrogenphosphate, pH 7.1 67:33	fluori-metric	200
SPE	C18	Gradient system: (A) 0.05 M n-HS (B) 95% MeOH + 0.05 M n-HS	UV 254 and 280 nm	65

[a] Solv. part.: solvent partitioning (classical extraction method); SPE: solid-phase extraction.
[b] MeOH: methanol, TEA: triethanolamine; n-HS: n-heptanesulphonate.

adopted to the purpose of the investigation. For routine analysis, which is often related to a great number of samples, SPE in combination with an isocratic HPLC system is preferable. Depending on the amount of alkaloid or if a selective detection is desired, fluorimetric detection is the method of choice. If one is interested in all alkaloids produced, fluorimetric detection is too selective. UV detection at at least two wavelengths gives information of peak purity and alkaloid chromophores. The recent developments in diode array techniques will soon make this type of detectors indispensable in the analysis of complex alkaloid extracts.

The identity of the alkaloids should further be confirmed by a second analytical technique. Usually, TLC combined with specific detection reagents is used. Detailed information on TLC [202] and HPLC [203] of alkaloids is available.

Due to their therapeutical importance, the *Catharanthus* alkaloids often serve as model compounds for analytical procedures. Drapeau et al. [204] described a method for the separation of dimeric indole alkaloids from monomeric indole alkaloid impurities. In reversed phase HPLC, the separation was based on differences in ion-pair formation characteristics of mono-

Table 11. Immunoassays for the determination of *Catharanthus* alkaloids and precursors in plants or cultures.

Alkaloid/precursor	Immunoassay	Ref.
ajmalicine	RIA	41, 207
serpentine	RIA	41, 207
vindoline	RIA	208–210
catharanthine	RIA	211
vinblastine	RIA	46
vincristine	RIA	212
vincristine	ELISA	213
secologanin	RIA	214
loganin	RIA	215

valent monomeric and divalent dimeric indole alkaloids. Marr et al. [205] used a mixture of ajmalicine, catharanthine and vincristine in a study on assessment of peak homogeneity in HPLC by computer-aided photodiode array detection; unresolved peaks could be quantitated by using a multiple component suppression method.

For SPE combined with HPLC still a reasonable amount of biomass is needed, 50–400 mg dry weight. For determination on cellular level, immunological techniques such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) have been developed, which supply very sensitive and often very selective assays. Because of the clinical importance of vinblastine and vincristine, RIA and ELISA methods have been developed to study their pharmacokinetic parameters [206, and references cited therein]. In plant cell biotechnology, these assays can be used for various sacreening techniques: distribution of an alkaloid within a plant, in different species or in different populations. Table 11 summarizes immunoassays developed for the determination of *Catharanthus* alkaloids and precursors in plants or cultures.

For the isolation of alkaloids from large amounts of biomass or medium, obtained from fermentations, solid phases can be used. So, a polystyrene resin (XAD-4) can be used for adsorption and selective removal of some alkaloids in aqueous solutions [216]. Because of the concentrating effect and the possibilities of selectively recovering products of interest, solid phase adsorption is an interesting method for alkaloid recovery from plant cell cultures.

7 Stability and preservation of cell lines

Many high-producing cell lines have proved to be unstable [147]. The reason for this is not known. Selection on growth by subculturing and chromoso-

Table 12. A cryopreservation procedure for *C. roseus* suspension cultures [after 220].

Preculture of 4-day-old cells	in nutrient medium supplemented with 1 M sorbitol for 6–20 h
Cryoprotection	in nutrient medium supplemented with 1 M sorbitol and 5% DMSO, in icebath for 1 h
Freezing	with a cooling rate of 0.5 °C per minute to −40 °C
Storage	liquid nitrogen
Thawing	rapid in a 40 °C water bath
Post-thawing treatments	transfer onto filter paper discs over nutrient media solidified with agar for 4–5 h; then transfer discs with cells to fresh medium of the same composition for regrowth

mal abberations may play a role here. Due to the instability, some techniques have been developed to maintain high-producing cell lines, which usually have been obtained by time-consuming selection procedures.

Cryopreservation has been applied successfully for *C. roseus* suspension cultures. This technique comprises the following stages: pregrowth, cryoprotection, freezing, storage, thawing and post-thawing treatments [217]. However, every step has to be optimized for every cell line (!), which makes this technique not readily available. Nevertheless, several successful procedures have been reported [217]. A protocol for *C. roseus* suspension cultures is presented in Table 12 [218–220]. The recovery of living cells was about 60% and they were able to produce indole alkaloids. Also in our laboratory some successful procedures for *C. roseus* cryopreservation have been developed [221].

Another technique for preservation of cell lines was suggested by Constabel et al. [36]: storage of regenerated shoots in dim light at 12–15 °C. This method has limited value, due to practical drawbacks, such as inefficient shoot formation etc. However, alkaloid production by initial callus and callus from regenerated shoots was fairly similar.

Morris [110] observed a preserved viability for suspension cultures up to 40 days by simply lowering the temperature to 10 °C.

For longer periods of preservation or for transportation purposes another three techniques have been described although exact data for *Catharanthus* are lacking: storage under paraffine oil at low temperature [222], undercooling of the cells [223] or storing the cells as semi-liquid suspensions [224].

8 Immobilization of plant cells

In nature, a single plant cell is always surrounded by other cells, a situation that offers protection and possibilities to co-operate and to communicate. In freely suspended cells these opportunities do not exist and this may influence both primary and secondary metabolism. To provide an artificial environment, plant cells have been immobilized in various matrices. Immobilization of plant cells has been reviewed [225, 226]. Viability and biosynthetic capacity must be preserved during immobilization. Viability of cultured *C. roseus* cells, estimated by plasmolysis, respiration and cell growth, could be retained using various matrices, e.g. alginate, agarose, agar, carrageenan etc. [225]. Vogel & Brodelius [227] used the non-invasive ^{31}P-NMR technique for studying the effects on cell metabolism of entrapment of cultured *C. roseus* cells in agarose and alginate. Phosphate metabolism and the cytoplasmic pH appeared unaltered. However, cell growth could damage the immobilized system. Majerus & Pareilleux [228] formulated a growth-limiting medium by optimizing 2,4-D and phosphate concentrations. The Ca-alginate entrapped cells were stabilized in this way, resulting in an enhanced alkaloid accumulation.

For industrial application it would be advantageous if the entrapped cells were easily attainable for substrates, precursors and/or co-enzymes. Furthermore, the formed products should be excreted into the medium. By permeabilization of immobilized plant cells by treatment with an organic solvent such as DMSO, 85–90% of the total intracellular ajmalicine was released without affecting cell viability to a great extent. This observation led to a model production system for ajmalicine by agarose or alginate entrapped cells and intermittent permeabilization [229]. Felix et al. [230] found that permeabilized *C. roseus* cells immobilized in agarose retained their enzyme activity far longer on storage than non-immobilized cells. They determined the activities of five enzymes from primary metabolism and one enzyme from secondary metabolism. Addition of the co-enzymes of the latter enzyme, cathenamine reductase, increased the yield of ajmalicine, 19-epi-ajmalicine and tetrahydroalstonine considerably. Recently, this permeabilization method has been applied to non-immobilized cultured cells of *C. roseus*. The degree of permeabilization of the tonoplast by DMSO could be quantified by measuring the P_i-efflux [231]. Tanaka et al. [128] permeabilized suspension cultured cells by using a medium with a high ionic strength. The enzyme 5′-phosphodiesterase was released with preserved cell viability.

The data obtained so far indicate that immobilization of whole plant cells could be of value for both biotransformation and de novo production

Table 13. Patents concerning cell and tissue cultures of *C. roseus* (source: Chemical Abstracts).

Year	Author	Application
1978	Zenk MH	ajmalicine and serpentine production by cultured cells
1981	Brodelius P, Zenk MH, Deus B, Mosbach K	production or transformation by immobilization of cells, protoplast or cell complexes
1982	Rosevear A, Lamke CA	biological production of chemical compounds; ajmalicine and serpentine are excreted in growth-regulator-free medium
1983	Miura Y	production of vinblastine by a callus culture
1984	Pfitzner U, Zenk MH	production of 3α(S)-strictosidine by immobilized strictosidine synthase
1984	Miura Y	after pretreatment with cellulase and pectinase, freezing and thawing, a callus culture is cultured to produce vinblastine again
1984	UK-Atomic-Energy	production of ajmalicine and serpentine by immobilization in modified polyacrylamide gel
1985	Tanaka H, Yajima M	release of 5′-phosphodiesterase by using high ion strengths
1986	Yokoyama M	bioconversion of hydroquinone to arbutin by callus cultures
1986	Miura Y, Hirata K	alkaloid production by organ cultures
1986	Constabel F, Kurz WGW, Eilert U	semicontinuous production by repeated elicitor treatment
1987	Kamata H	production of alkaloids by *A. rhizogenes* transformed plants
1987	Rosevaer A, Hislop I, Lambke CA	production of serpentine in a flat biochemical reactor (sheet of PUR)
1987	Rosevaer A, Simmons R	continuous flow serpentine production by immobilized cells; the culture medium was recirculated through a C-18 cartridge

systems. The use of naturally immobilized culture systems, as reported for *Cinchona* [232], in which the cells are aggregated and differentiated, may offer a valuable alternative. Furthermore, immobilization can be used to overcome the problem of shear stress, if it exists, in fermentation processes.

9 Economical aspects

The dimeric indole alkaloids represent a market of economical interest: vinblastine ($\$1{,}000{,}000\,kg^{-1}$, volume $12\,kg\,year^{-1}$) and vincristine ($\$3{,}500{,}000\,kg^{-1}$, volume $1\,kg\,year^{-1}$) [233]. Despite all efforts, nobody succeeded to produce these alkaloids by suspension cultures of *C. roseus*. Ajmalicine, a monomeric indole alkaloid of pharmaceutical interest ($\$2000\,kg^{-1}$, volume $5000\,kg\,year^{-1}$) can be produced by suspension-cultured cells, but the yield ($0.3\,g\,l^{-1}$ [411]) is still far from being economically feasible. However, the production of penicillin became 3600 times more efficient in 40 years of intensive research. As plant cell biotechnology is just in its infancy, a considerable improvement of the efficiency of plant cell cultures may be expected. The increased efforts are, for example, illustrated by the number of patents concerning the production of alkaloids by plant cell and tissue cultures. The number increased substantially the last years. Table 13 summarizes some patents concerning *C. roseus*. These patents comprise a large spectrum of applications, e.g. production of alkaloids, biotransformations of xenobiotics, extraction procedures etc.

In the near future the production of catharanthine by fermentation and the bioconversion of catharanthine and vindoline to dimeric indole alkaloids may probably be of economical interest.

10 Concluding remarks

During the last two decades the knowledge of and the insight in the production of secondary metabolites by plant cell and tissue cultures has increased substantially, which immediately resulted in the development of commercial production processes. For *C. roseus* in particular, more insight has been obtained on the regulation of the secondary metabolism and the biosynthesis of the alkaloids. Although techniques have been brought into practice, much of the necessary fundamental knowledge is lacking. Items which need further investigation to fill up some gaps in our understanding of the process of product accumulation, are amongst others:

— cell line stability,

— regulation of the terpenoid and the alkaloid pathway,
— developmental regulation,
— catabolism of alkaloids,
— excretion, uptake and storage mechanisms.

Furthermore, more experience with large-scale culturing of plant cells is needed.

The outcome of that research may lead to a commercial exploitation of cell and tissue cultures of *C. roseus*.

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