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Exploiting plant cell culture for natural product formation

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Summary

The initiation of callus cultures and *in vitro* cultivation of plant cells, even in large bioreactors, has become a routine task. Despite the fact, that permanent cell and organ cultures can produce a whole range of small natural compounds (SNAPs) used in medicine, only a few could be produced at commercial scale. However, plant cell cultures provide very useful systems to study the biosynthetic pathways leading to SNAPs at the enzyme and gene level. They turned out to be 'a pot of gold' for those chasing the enzymes and genes involved in natural product formation. The use of genetically modified yeast and bacteria for the production of SNAPs is an emerging technique that will take research into the coming decades. This review contemplates and revisits developments in the field of using plant cell and tissue culture as tools to elucidate SNAP formation and means to produce bioactive plant natural products over the past 40 years alongside my own work.

Keywords: Secondary metabolism, Specialized metabolism, Cell suspension culture, Bioreactor, Elicitation, Biochemical pathways, Natural products

Introduction

A multitude of small natural products (SNAPs), such as alkaloids, small peptides, isoprenoids, phenolics, and polyketides generated by plants are used as pigments, food additives or pharmaceuticals. Some of them are bioactive in an ecological or co-evolutionary sense. In an average plant about 90% of the individual products formed can be characterized as SNAPs (Firn, 2010) although some are only produced in trace amounts.

As early as the 1970s my interest in the biochemistry, physiology, chemistry, and analysis of SNAPs was sparked and still I regard each discipline as equally important when dealing with SNAP formation. At that time SNAPs were sometimes regarded as 'flotsam and jetsam on the metabolic beach' (Haslam, 1986). The model of chemical co-evolution eventually brought them into focus (Hartmann, 1996) and the vast structural diversity kindled much enthusiasm and attracted many researchers. Jones and Firn (1991) introduced the screening hypothesis in order to explain the existence of hundreds of thousands of individual SNAPs. Their hypothesis is based on the assumption that organisms making SNAPs face the same challenges as pharmaceutical companies trying to find new drugs. Only recently, Noda-Garcia et al. (2018) have offered an integrative metabolite-enzyme co-evolution hypothesis proposing that metabolite pools change alongside the appearance of newly evolved enzymes. If an emerging SNAP provides a selective advantage for its producer, enzymes will be recruited or shaped resulting in improved synthesis. Any of these hypotheses try to explain the occurrence of the multiplicity of SNAPs. These hypotheses rather complement than contradict each other and may even be merged.

In the 1970s reports on the biosynthesis of SNAPs were still limited. How did we study SNAP biogenesis in these days? Beadle and Tatum (1941) used *Neurospora crassa* as a model organism to elucidate biochemical pathways. They established that individual gene mutations were responsible for single metabolic steps ('one gene-one enzyme' hypothesis) by constructing mutant strains requiring specific amino acids or vitamins for their survival. This approach, however, could not be successfully employed in elucidating the pathways leading to SNAPs. They are not necessary for the survival of the producing cell, lethal mutants could not be generated and their deficiencies then repaired by adding appropriate precursors. The only change in phenotype that could easily be observed was pigment formation (see *Selection and characterization of biochemical mutants*). Many of the pathways shown in the textbooks were based on chemical plausibility or studies using radiolabeled compounds assumed to be precursors in a particular pathway. Experiments were usually carried out with whole plants or excised plant organs. One particular booklet (Bu'Lock, 1965), translated into German and also slightly edited by Wolfgang Barz and Hans Grisebach (Bu'Lock, 1972), was sufficient to convince me that the field 'biosynthesis of natural products' is large enough to nurture generations of scientists.

Traditionally SNAPs are isolated from intact plants since chemical synthesis proved to be complicated and expensive. Production of SNAPs by plant cell cultures could provide an alternative source of SNAPs. Articles reviewing this idea generally begin with the statement that hundreds of plants have been used to establish cell cultures. Among these plants are many endangered species and a lot of plants containing compounds with, *e.g.*, anti-cancer, neuroprotective or anti-malarial activities. Most of the reviews repeat the following statements like a mantra: as an alternative way to produce SNAPS, cell and tissue culture have the advantages that (1) fields can be freed up for growing crops instead, that (2) plant cell culture systems are not affected by weather and season changes and that (3) their metabolism can be manipulated to maximize production. One may challenge all of these statements and find arguments contradicting them. For example, in Germany medicinal and aromatic plants are cultivated on only 13,000 ha (IVA, 2019) whereas cereals occupy about 6,000,000 ha (AGRAVIS, 2019). These figures warrant criticism against the first argument. The overwhelming amount of literature on plant cell cultures and their use in SNAP formation made it impossible to write a non-biased review covering the developments over the past 40 years. A literature search conducted on the Google Scholar Website using the search terms "natural products" and "plant cell suspension" and covering the years 2015 - 2019 yielded 386 hits, among them 52 reviews or book chapters (*e.g.*, Dias et al., 2016; Ochoa-Villarreal et al., 2016; Yue et al., 2016). A recent meta-analysis study came to the conclusion that plant cell technology is the best strategy for production of natural-based drugs (Davoodi et al., 2019). Contemplating on this astonishing statement and the plentitude of quite recent reviews I here tried to put my own experiences in the field in a methodological and historical context and give my very own assessment of the state-of-the-art (Fig. 1).

I took the book entitled 'Plant Tissue Culture and Its Biotechnological Application' (Barz et al., 1977) as my personal starting point. The proceedings, edited by Wolfgang Barz (Münster), Ernst Reinhard (Tübingen), and Meinhart Zenk (München), documented the

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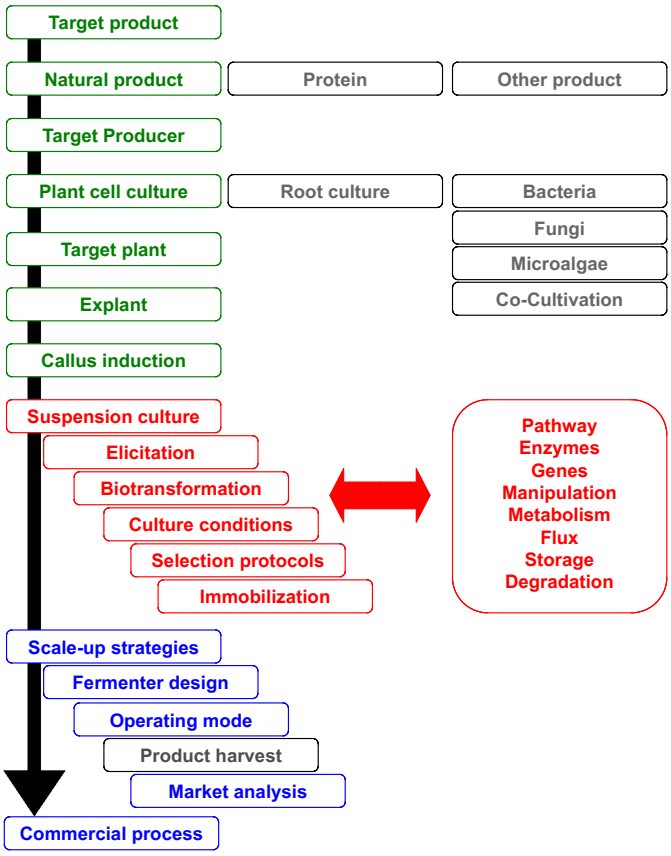

Fig. 1: Strategies for the alternative production of natural products (SNAPs). Special emphasis is only put on plant cell suspension cultures. The latest results gained from the cultivation of genetically transformed roots are not taken into account. The use of microorganisms, including microalgae, in the production of plant-borne SNAPs is not addressed in a comprehensive way here. Neither are methods for product isolation and release. Other products, like proteins, are not addressed in this review. The areas not reviewed in detail are shown in grey. Issues related to large-scale culture and commercialization are shown in blue. Methods using plant cell suspension cultures for pathway elucidation are shown in red. The black arrow indicates the path from a target product to an industrial process using plant cell suspension cultures. The arrow also defines the structure of this review.

state-of-the-art in the field at the time. Well-reputed scientists from all over the world shared their experiences and the titles of their contributions could still be used to structure a review article today. In these proceedings ALFERMANN et al. (1977) put forward the results of Ernst Reinhards's group in Tübingen in a contribution entitled 'Biotransformation of Cardiac Glycosides by Plant Cell Cultures' and I was lucky enough to get a postgraduate position in this particular group in 1983 where I got the chance to combine in my research analytical, plant physiological, biochemical and biotechnological aspects of plant cell culture (see *Growth of plant cells in bioreactors*). Plant cell cultures are nowadays also considered to produce recombinant proteins, such as antibodies. However, this issue will not be touched here and the reader is referred to other reviews dealing with various aspects of protein production by suspension-cultured plant cells (FISHER et al., 1999; HUANG and MCDONALD, 2009; TEKOAH et al., 2015).

Initiation of plant cell cultures

In 1934, WHITE and GAUTHERET independently demonstrated that plant cells and tissues can be grown and propagated for long periods of time in complex media. By including indole-3-acetic acid and vitamins in his media, GAUTHERET (1934) extended the culture period of callus to 18 months by including indole-3-acetic acid and vitamins in his media and he was able to subculture the tissue aseptically. ROUTIEN and NICKELL (1956) described the submerged cultivation of tissues of several plant species and were granted the first patent for the cultivation of plant cells *in vitro*. Today, the initiation of plant cell and tissue cultures and cultivation of plant cells and tissues *in vitro* has become routine. Cell behavior can be triggered with phytohormones and even regeneration of intact plants became possible. A number of chemical factors like media components, phytohormones and pH as well as physical factors like light, aeration and temperature, which can affect plant cell growth *in vitro* have been investigated. Several textbooks, proceedings and lab manuals have been published over the past four decades to cover the topic more comprehensively than possible here (REINERT and YEOMAN, 1982; ALLEN, 1991; GAMBORG and PHILIPPS, 1995; COLEMAN et al., 2003; LOYOLA-VARGAS and OCHOA-ALEJO, 2018). Under certain conditions plant cells can grow as dedifferentiated callus masses. Culture media usually include salts of inorganic macro-nutrients (N, P, S, K, Na, Ca, Mg) and micro-nutrients (I, Ni, Fe, Cu, B, Mn, Co) as well as vitamins and an appropriate carbohydrate source. The most common media for plant cell culture are those devised by MURASHIGE and SKOOG (1962), GAMBORG et al. (1968), SCHENK and HILDEBRANDT (1972), and WHITE (1934). Under certain conditions plant cells can grow as de-differentiated callus masses. After several medium changes these cell masses can be used to study various aspects of SNAP formation and eventually plant cell culture turned out to be an excellent system to understand and manipulate SNAP formation. Application of precursors or inhibitors is simple and analysis is facilitated by the fact that bulk compounds, such as phenolics or chlorophyll are not as abundant as, for example, in plant leaves. Long-distance transport does not happen in plant cell culture, an obvious advantage for studying SNAP metabolism. In intact plants precursors can be transported, sequestered and/or modified in various ways prior to reaching the site of SNAP biosynthesis. Precursors may even be degraded to very small molecules, such as acetate, which in turn can be used 'second hand' for SNAP formation. For example, the discovery of the non-mevalonic acid terpenoid pathway (LICHTENTHALER et al., 1997) supported the view that studies with radio-labeled precursors can lead to ambiguous conclusions. This and other examples demonstrate that even 'trodden' pathways like those depicted in generations of textbooks, do need confirmation by using modern techniques (see *Chasing enzymes of SNAP formation in plant cell cultures*). Plant cell cultures are an appropriate tool to do so provided they express the pathway to be examined.

SNAP formation in callus cultures

Extensive lists of plant cell cultures accumulating bioactive SNAPs of most of the biosynthetic classes known were compiled (MULABAGAL and TSAY, 2004; KARUPPUSAMY, 2009). Many of the reports cited used callus cultures, which provide a reliable and easily accessible material for conducting biosynthetic studies. Callus cultures of *Portulaca grandiflora* Hook. were the first cell cultures to catch my interest. They produce betalains and depending on their biosynthetic capacity they accumulate red and yellow pigments possessing a betalamic acid moiety. Betalamic acid is derived from L-tyrosin needing a 4,5-dioxygenase, which has been isolated from *P. grandiflora* (CHRISTINET et al., 2004). L-tyrosine is also a precursor of Cyclo-DOPA and the conjugation of betalamic acid with cyclo-DOPA yields betanidine. We found that betanidine biosynthesis is favored in the dark and that several catecholamines, including adrenaline, also accumulate in darkness. Methyldopa, a drug developed for the treatment of high blood pressure, inhibited

catecholamine biosynthesis in *P. grandiflora* callus (Endress et al., 1984). Some questions began to puzzle me: (1) Do plants somehow respond to the mammalian stress hormone adrenaline? (2) Could it be a 'secondary' metabolite in plants but a 'primary' one in humans? (3) Is the metabolism associated with its formation 'secondary'? Much later, Firn and Jones (2009) stressed that the same biochemical rules apply for all pathways, albeit to a different extent because they operate under different evolutionary constraints. There is no theoretical basis for separating metabolisms into 'primary' and 'secondary' and I decided to avoid these terms where ever possible. Actually, only a few basic metabolic principles provide access to the majority of SNAPs: (1) starting from biogenic amines Mannich-type reactions lead to a variety of alkaloids ('alkaloid pathways'), (2) the isopentenyl-diphosphate/dimethyl allyl diphosphate system provides building units for a large number of terpenoid skeletons ('terpenoid pathways'), (3) phenolic acids derived from phenylalanine and tyrosine provide building blocks for a large number of phenolics ('phenylpropanoid pathway'), (4) malonyl-CoA derived from acetyl-CoA provides a universal and sequential C2-group donor used in the formation of a plentitude of polyketides ('polyketide pathway'). (5) Small peptides can be produced via the processing of ribosomally produced precursors or with the help of nonribosomal peptide synthetases (Barber et al., 2013).

The rich diversity of SNAPs found arises from modifications by only a limited number of chemical substitution types, such as hydroxylation, glycosylation, acylation, prenylation, and methylation. The formation of members of all the biogenetic groups mentioned have been investigated in plant cell cultures (see *Chasing enzymes of SNAP formation in plant cell cultures*).

Selection and characterization of biochemical mutants

Callus can be grown for months and even years. It is, however, a quite heterogeneous population of cells. Small clumps or aggregates can be picked from these cell masses to develop virtually homogeneous clones of fast growing viable cell cultures. Several screening and selection protocols have been developed (Widholm, 1977; Berlin and Sasse, 1985) and are still valid today. Elite cell lines can be obtained by selection following various strategies including macroscopical, microscopical and chemical inspection. Cell-aggregates or protoplasts can be used as the starting material for selection (Dix, 1990; Kreis et al., 1992). Variation can be enhanced employing induced physical or chemical mutagenesis (Ahloowalia et al., 2001; Donini and Sonnino, 1998). Selection can be achieved easily if the product of interest is a pigment. For example, anthocyanin-rich *Euphorbia milii* Des Moul. cells and cell clusters were selected and a cell strain was established producing 7 times as many anthocyanins as the initial cell culture (Yamamoto et al., 1982). Screening and selecting high-producing cell lines was not always successful and 'recalcitrant' cell cultures were the rule rather than the exception (Kreis, 2003). Cell cultures of *Papaver somniferum* L., for instance do not produce quantities of morphinanes worth considering. Low or no apparent productivity of alkaloids in plant cell cultures was explained by insufficient cell differentiation (Farrow et al., 2012). This is supported by the fact, that non-producing plant cells may recover their biosynthetic capacity during morphogenesis. For example, in a *Duboisia leichhardtii* (F. Muell.) F. Muell. callus scopolamine production and accumulation was restored after root induction, but not after shoot induction (Yamada and Endo, 1984). Root cultures, by the way, have also turned out to be suitable systems for producing SNAPs and for studying SNAP formation (Oksman-Caldentey and Arroo, 2000; Srivastava and Srivastava, 2007; Yue et al., 2016) but this topic will not be reviewed here.

Plant tissues, when cultured *in vitro,* show genetic instability even without mutagenic treatment (Stafford, 1991). Cells can become habituated and ultimately exhibit hormone-autotroph callus growth which may explain the loss of regeneration potential after a prolonged callus phase. For example, in callus of *Digitalis mariana* ssp. *heywoodi* (P. Silva & M. Silva) Hinz morphogenesis could still be induced after 24 months but not after 32 months. Cardenolide production was impaired in plants regenerated after several months of cultivation as callus (Kreis et al., 2014). The variations occurring in plants regenerated from tissue culture or in permanent plant cell culture were termed 'somaclonal variation' (Larkin and Scowcroft, 1981), which will also occur in permanent plant cell culture. The 'apparent' stability (meta-stability) of a plant cell culture 'strain' may be the effect of a strict subculture regime (Ulbrich et al., 1985; Fowler, 1988) rather than the result of the clonal origin of the cells. The loss in productivity reported for many plant cell suspension cultures might be caused by rapidly dividing cells overgrowing the SNAP accumulating cells (Stafford, 1991). This phenomenon may constitute a problem for the establishment of a commercially viable plant cell culture process (see *Commercialization of plant cell culture processes*).

SNAP production using plant cell suspension cultures

Callus culture is not well suited as a method to commercially produce SNAPs. It cannot be used for the large-scale production of secondary metabolites because of slow growth rates and the lack of suitable bioreactor systems for their cultivation. However, calluses might disintegrate into small cell aggregates when submerged in liquid media yielding cell suspension cultures. Actually, most of the physiological and biochemical experiments designed for elucidating metabolic pathways have been carried out with suspension-cultured cells. The cells are kept in flasks on appropriate shakers to allow for aeration under various light-dark regimes. Reports on the production of SNAPS by plant cell cultures generally used small tissue samples grown in standard shake flasks allowing for easy sampling and additions to the suspensions. Growth curves are usually determined during process optimization. For this purpose, we introduced a simple, sample-free method to allow for rapid estimation of cell growth in Erlenmeyer flasks (Blom et al., 1992). The method proved to be very suitable to determine the increase of cell mass during each phase of the growth cycle and it is still used in our laboratory today.

Because metabolic activity is a function of the surface of an organism, growth and production rates of plant cell aggregates are much lower than those of microbial cells. In microbes secondary metabolite production is not necessarily associated with biomass production. Products are generally released into the bathing medium, production rates are high and production phases are short. The growth of plant cell cultures is quite slow, metabolite production is low and the products are usually stored in the cells (Scragg, 1995). For example, rosmarinic acid accumulates to 36% DW in cultured cells of *Salvia officinalis* L. (Hippolyte et al., 1992), and berberine (Kobayashi et al., 1988) or its derivative jatrorrhizine (Breuling et al., 1985) can reach about 10% of the cells' dry mass. Although the idea of using plant cell cultures for the production of SNAPs is reasonable and even hyped in the 1980s and 1990s, tables of successes are still quite short and should be regarded with caution as far as productivity claims are concerned (see *Commercialization of plant cell culture processes*). The list of plant cell cultures not producing the compounds of interest in amounts large enough to consider commercialization is much longer (Kreis, 2007).

Elicitation of SNAP formation in plant cell cultures

Though all approaches to produce morphinanes in plant cell cultures so far have failed (see *Selection and characterization of biochemical*

mutants), compounds sharing the same precursors and intermediates as the morphinanes, such as benzophenanthridines, can accumulate in rather large amounts. It was demonstrated that the production of sanguinarine was even enhanced by adding preparations from fungal mycelia (EILERT and CONSTABEL, 1986) and many studies have shown, that SNAP production can be provoked by exposing cultured plant cells to fungal cell wall fragments (BARZ, 1988; PARK et al., 1992; GUNDLACH et al., 1992). Preparations like these belong to the so-called 'elictors', exogenous molecules inducing physiological abnormalities that are often associated with plant pests. A broader definition of them includes exogenous and endogenous elicitors, as well as compounds released from plants in direct response to the pathogen. The label 'chemical elicitors' is now even used for substances like methyl jasmonate, methyl salicylate or benzoic acid, which are plant hormones and not elicitors in the original sense (PATEL and KRISHNAMURTHY, 2013). For example, methyl jasmonate 'elicitation' induced paclitaxel and related taxane production in *Taxus* cells (TABATA, 2004; see *Commercialization of plant cell culture processes*). The elicitor approach was not successful in all cases: morphinane biosynthesis, for example, could not be elicited as yet. Anyway, the application of elicitors (in the broader sense) has been considered as one of the most effective methods to improve the synthesis of SNAPs in plant cell cultures and elicitors can also be used to increase yield in field-grown plants (BAENAS et al., 2014) demonstrating the impact of basic research conducted with plant cell cultures.

Biotransformation of SNAPs by plant cell cultures

Suspension-cultured cells can be used in so-called biotransformation processes. Biotransformation studies have been carried out with a view to (1) produce new chemicals, (2) produce known chemicals more economically, (3) investigate the metabolic fate of xenobiotics, and (4) elucidate metabolic pathways (REINHARD and ALFERMANN, 1980; SUGA and HIRATA, 1990; STEPAN-SARKISSIAN, 1991; GIRI et al., 2001). For example, none of the cell cultures investigated so far was able to produce scopolamine, a drug used in urinary incontinence and motion sickness, but the ultimate biosynthetic step, the conversion of hyoscyamine into scopolamine was realized in transgenic tobacco cell cultures (MOYANO et al., 2007). In one of our own more recent biotransformation studies cell suspension cultures of *Digitalis lanata* Erh. were fed with 21-*O*-acetyl-deoxycorticosterone and three new pregnane compounds were formed and their structures elucidated (type (1) approach; PÁDUA et al., 2012). In a type (2) approach, the commercially unavailable glucoevatromonoside, a natural cardenolide inducing cancer type-specific cell death (SCHNEIDER et al., 2018), was produced in *D. lanata* cell cultures (MUNKERT et al., 2017).

Already in the 1970s *D. lanata* cells cultivated *in vitro* were shown to convert digitoxin derivatives into digoxin derivatives (REINHARD and ALFERMANN, 1980). From this line of research a cell culture process has emerged where viable amounts of β-methyldigoxin are generated from β-methyldigitoxin, a semi-synthetic cardenolide with almost no side reactions. The β-methyldigoxin process was successfully scaled up to a volume of 200 L (ALFERMANN et al., 1983; REINHARD et al., 1989).

At that stage the reason why digitoxin could not be used to establish a putative digoxin process was unclear. Storage, transport and sequestration had not be considered to understand the cellular organization of the processes involved in cardenolide biotransformation. The vacuole was assumed to be a storage site for cardenolides but it could not be explained why some of the biotransformation products were released into the bathing medium while others remained in the cells. Eventually, procedures to isolate intact vacuoles from plant cells cultivated *in vitro* became available and were used to investigate SNAP formation and storage (DEUS-NEUMANN and ZENK, 1984; KREIS and REINHARD, 1985; MENDE and WINK, 1987; HOPP and SEITZ, 1987). After feeding digitoxin to suspension-cultured *D. lanata* cell we were able to demonstrate that only cardenolides possessing a terminal glucose moiety were stored in the vacuole. Moreover, we found that cardenolides without a terminal glucose enter the cells by simple diffusion, whereas those containing the glucose tag (termed 'primary glycosides') are taken up actively (KREIS and REINHARD, 1987). Once accumulated, the primary glycosides were retained by the cells even if cyanide was added to the cell suspensions indicating that no energy was required to keep the primary glycosides stored. Of course, the enzymes involved in cardenolide biosynthesis had to be identified and characterized (see *Chasing enzymes of SNAP formation in plant cell cultures*). Finally, a model featuring the mechanisms involved in cardenolide biotransformation and storage was proposed to help with the development of new biotransformation processes on a large scale (KREIS et al., 1992; see *Growth of cell cultures in biorectors*).

Chasing enzymes of SNAP formation in plant cell cultures

To characterize enzymes and genes of a metabolic pathway the target enzyme needs to be purified, the protein sequenced and the appropriate nucleotide primers synthesized. This classical approach benefitted from some of the characteristics of plant cell cultures which can be cultivated under standardized conditions and manipulated in a multitude of ways. Protein extraction and purification of SNAP forming enzymes, which may occur in low concentrations only, turned out to be very straightforward and efficient (ZENK, 1991). For example, ajmalicine was the first alkaloid whose biosynthesis was elucidated at the enzyme level using *Catharanthus roseus* (L.) G. Don cell suspension cultures (ZENK, 1980). Much of the biosynthetic pathway leading to morphinane alkaloids was investigated using plant cell cultures (HAGEL and FACCHINI, 2013). Others and we isolated the enzymes involved in cardenolide biotransformation (KREIS et al., 1998; see *Biotransformation of SNAPs by plant cell cultures*) and also investigated early steps of the cardenolide pathway. For instance, we isolated the Δ5-3β-hydroxysteroid dehydrogenase (*Dl*3βHSD) from *D. lanata* tissues (SEIDEL et al., 1990). This enzyme catalyzes the formation of pregnenolone from progesterone. A more recent discovery is that *Dl*3βHSD, unlike other known mammalian enzymes, only has oxido-reductase activity but not Δ^5-Δ^4-ketosteroid-isomerase activity (MEITINGER et al., 2016). Progesterone is further metabolized to 5β-pregnane-3,20-dione by the progesterone 5β-reductase (GÄRTNER et al., 1994) which was proposed to be the first regulatory 'key' enzyme in the 5β-cardenolide pathway. However, later it was shown that the enzyme was active and the respective gene expressed in suspension-cultured cells (not capable of producing cardenolides) and cardenolide-free tissues of *D. lanata* (ERNST et al., 2010).

Enzymes at the start of a biosynthetic pathway are sometimes described as 'key' enzymes, but they are not necessarily rate-limiting for production. The bottleneck(s) often occur further down the pathway. Rate-limiting or pathway-pivotal enzymes do not necessarily have to catalyze spectacular reactions but may instead only adorn scaffolds with small structural elements. This was nicely demonstrated in berberine-producing *Thalictrum* cell cultures. Zenk's group and later Kutchan and co-workers (FRENZEL and ZENK, 1990; FRICK and KUTCHAN, 1999) tried to ascertain why some cell culture strains were producing berberine-type alkaloids while others failed. They found that most of the enzymes necessary for the formation of protoberberines were present in non-producing cells but the full set of all four methyltransferases involved in protoberberine biosynthesis was only expressed in producing cells. Methyl groups attached to a scaffold by *S*-adenosyl-*L*-methionine-

dependent methyltransferases can decide the destiny of a precursor. The precursor might not be funneled into the target pathway if the substitution pattern facilitates inappropriate condensation reactions due to the lack of protecting groups. Meinhart Zenk once stated that "the development of plant cell cultures for the study of the biosynthesis of secondary metabolites in the 1970s revolutionized the field. It became possible to identify, characterize and ultimately, in specific cases, to purify the biocatalysts involved in individual transformations. The precise knowledge of the biosynthetic pathways provided by the identification of these enzymes, of the stereo- and substrate-specificity of the reactions they catalyze and of the sequence of these reactions *in situ* opened new fields for study in plant sciences" (Zenk, 1991) and indeed, for more than 30 years plant cell cultures have become a central tool to study SNAP formation.

It turned out that SNAP formation appears to utilize multidimensional grids rather than linear pathways and that various factors can control the flux through a biosynthetic grid. These factors are: (1) enzyme amount/activity, (2) metabolite inhibition/stimulation, (3) pathway competition, (4) co-factor availability, and (5) compartmentalization (Verpoorte et al., 2000). Potential flux limitations can be investigated by feeding different precursors to cultured plant tissues or cells. The question arose of how specialized SNAP metabolism actually is because the multitude of metabolites found in living organisms does not correspond with the small number of metabolic genes identified in the various genome studies (Schwab, 2003). Convergent, parallel, and divergent evolution can be seen in metabolic processes (Pichersky and Gang, 2000; Delgoda and Murray, 2017). Gene duplication (Ohno, 1970) and divergence to more specialized enzymes may be important for SNAP formation (Ober, 2005). However, enzymes with a relaxed substrate specificity increase the possibility of producing a greater number of new chemicals (Weng and Noel, 2012; Kreis and Munkert, 2019). Enzymes may compete for common intermediates. A particular enzyme may catalyze a series of parallel reactions involving structurally related substrates. There are still metabolic uncertainties in every step of a given pathway or in a metabolic grid. The flux to a certain compound is predominantly decided by grid architecture. At enzymatic level only a few pathways/grids have been studied extensively enough to allow for serious pathway engineering (see *Outlook*). In most cases SNAP pathways are still far from being elucidated. As an example the biosynthesis of tropic acid and the ester alkaloid formation in scopolamine biosynthesis has been the subject of continued discussion and remains an unresolved issue (Qiu et al., 2018).

Eventually SNAP formation research slipped into the genomic and post-genomic area. Screening of cDNA or genomic libraries and the use of PCR approaches made it possible to identify, isolate and clone genes involved in SNAP formation. It became feasible to express them heterologously in microorganisms with the aim to obtain enough protein for characterization and structure elucidation. We also contributed to this field by elucidating the crystal structures of two progesterone 5β-reductases (Egerer-Sieber et al., 2006; Schmidt et al., 2018). During this phase plant cell cultures became nearly obsolete. This is underlined by the fact that about 10 years ago the DSMZ (German Collection of Microorganisms and Cell Cultures) maintained more than 700 different plant cell lines from more than 80 different plant families (Kreis, 2007). In April 2019, 41 plant cell lines were still provided as growing or cryopreserved cultures (DSZM, 2019a) but now (DSMZ, 2019b) the catalogue of strains does not offer plant cell cultures any longer.

Either way plant cell cultures with their limited potential and complexity turned out to be a good system for metabolome studies. The metabolome is defined as the final downstream product of the genome and consists of all metabolites in a cell, tissue or organism. A SNAP metabolome may be defined as a part of the metabolome related to a specific group of SNAPs. The 'taxane metabolome' of a *Taxus* cell culture will hence comprise all taxanes, including taxa-4,11-diene, formed by the initial action of a diterpene cyclase from geranyl-geranyl diphosphate and the highly complex paclitaxel that may be regarded as the end product of the taxane pathway. Over 400 taxanes described so far are represented in this constrained metabolome (Croteau and Ketchum, 2006) but most of them are not part of the actual paclitaxel biosynthetic pathway(s). Taxol biosynthesis requires 19 enzymatic steps, but so far only 15 enzymes have been characterized (McElroy and Jennewein, 2018). It involves eight oxidation steps, two CoA esterifications, *N*-benzoylation, five acetyl/aroyl transferase steps, a C4β,C20-epoxidation reaction, and a phenylalanine aminomutase step. Understanding the relationship among all intermediates in the metabolic grid may provide clues for metabolic engineering of plant cell cultures for increased taxol production. It has been stated that to increase taxol yields in *Taxus* cell cultures these „numerous and apparently diversionary taxoid biosynthetic side-routes and dead-ends" have to be taken into account (Croteau et al., 2006; see *Commercialization of plant cell culture processes*).

Plant cell cultures have also been used to decipher multiple levels of cellular control of lignin deposition, composition, structure, and quantity. In this case, plant cell cultures have provided insights into the lignification processes that could not have been easily obtained by using whole plants (Pesquet et al., 2019).

Growth of plant cell cultures in bioreactors

Already in the 1960s John D. Bu'Lock (see *Introduction*) saw that biotechnology (at that time usually referred to as 'fermentation technology') would be the next big development to happen for the production of SNAPs. Large scale cultivation of plant cells in bioreactors was the obvious next step (Kreis and Reinhard, 1989; Scragg, 1995; Sharma and Shazad, 2013; Eibl and Eibl, 2008). In the 1970s pneumatically agitated air-lift bioreactors were advertised and routinely used for the cultivation of plant cell aggregates that were assumed to be highly shear-sensitive (Verpoorte et al., 2002). Today many different designs and configurations of bioreactors for plant *in vitro* systems are available. The most common type of reactor for growing plant cells is the stirred-tank bioreactor, a mechanically agitated vessel. This type of reactor uses impellers for gas-liquid transfer and mixing. Though bioreactors are scalable, reproducing the physical environment (shear, mixing, gas transfer) is often difficult (e.g., Scragg, 1991). Big challenges in bioreactor design are the efficient illumination of phototrophic cell cultures (Huang et al., 2017) and the use of disposable bioreactors (Eibl and Eibl, 2008).

Some engineering aspects will have to be addressed before citing examples of processes developed (see *Commercialization of plant cell culture processes*). The 'operating mode' refers to how the nutrient and product streams are supplied to or removed from a culture. When all nutrients are supplied from the beginning the operating mode is termed (1) batch culture. In a (2) fed-batch operation one or more nutrients are added to the bioreactor during cultivation, either in batches or continuously. Fed-batch mode is more suitable than standard batch cultivation if a nutrient or precursor is detrimental to the cells when supplied all at once. In (3) repeated fed-batch operation a substantial portion of the spent medium or the cell suspension is withdrawn and replaced with fresh culture medium. (4) Two-stage batch mode involving a change of culture media is an option to allow for rapid growth in the first stage and product synthesis in a second stage. For example, a two-stage process for the production of deacetyllanatoside C was designed and it was demonstrated, that this process can be run in a semi-continuous mode (Kreis and Reinhard, 1990). (5) Chemostat operation refers to a fermentation mode in which fresh medium is continuously fed to the bioreactor and a stream of cell suspension is continuously withdrawn for pro-

duct harvest or metabolite analysis. In (6) perfusion operation fresh medium is added to the vessel continuously and only spent medium is withdrawn. For example, berberine production using *Coptis japonica* (Thunb.) Makino cells was improved by a factor of 6.5 when a continuous turbidostat set-up was used instead of the batch mode (MATSUBARA et al., 1989).

The perfusion reactor is also appropriate for immobilized producers. Immobilization is a technique which traps catalytically active cells or enzymes in a matrix that prevents them from entering the liquid phase. Plant cells immobilized in alginate or on porous glass beads have been used for the *de novo* biosynthesis of valuable SNAPs as well as for biotransformations (YEOMAN, 1987; RAMACHANDRA RAO and RAVISHANKAR, 2002; BRODELIUS, 2018). This issue will not be elaborated further here and the reader is referred to the respective reviews.

Commercialization of plant cell culture processes

During the dynamic 1980s I had the opportunity to visit Japan and some of the companies using plant cell cultures for the production of SNAPs already then. Mitsui Petrochemical Industries were about to establish processes for *Vinca* alkaloids, *Rubia* dyes, berberine, and shikonin, while Shiseido focused on an arbutin process using *Catharanthus roseus* (L.) G. Don cell cultures. Nitto Denko produced ginsenoside-rich biomass using *Ginseng* root cultures and Nippon Paint and Sanei Chemical ran their own plant cell culture projects. Optimization of berberine production from *Coptis japonica* (Thunb.) Makino cells resulted in a yield of 3.5 g/L after a scale up to 4 m^3 (MATSUBARA et al., 1989). However, as recently pointed out by LANGE (2018) the introduction of plant tissue culture-derived fine chemicals to the market has been confined to few examples, including the 1988 'one-hit wonder' shikonin (FUJITA, 1988) and the 'showpiece' paclitaxel. Phyton Catalytic (now Phyton Biotech) began the scale-up of cell suspension cultures of *Taxus chinensis* (Miq.) de Laub. in the 1990s. After extensive studies, a large-scale culture process was developed where the addition of the 'elicitor' methyl jasmonate increased the productivity to 23.4 mg L^{-1} d^{-1} (KETCHUM et al., 1999). A production volume of 75 m^3 was established and in 1995 the plant cell culture process was licensed to Bristol-Myers Squibb. Eventually several new tissue culture processes for cytostatic taxanes emerged (CHOI et al., 2002; MCELROY and JENNEWEIN, 2018).

It seems appropriate to comment on the profitability of plant cell culture at this point. First of all there has to be a sufficiently large market for the product. The most important parameter for possible commercialization of a cell culture process is its productivity. There are many different definitions of productivity. We defined it as the amount of product formed per volume and time. This figure is dependent on growth and product formation rates. It takes into account unproductive phases and the time required for preparation and follow-ups of a production run. It was estimated that the wholesale kilo price must be between 250 U.S. $ and 8,000 U.S. $ for any plant cell culture process with a production rate between 0.025 to 1.0 g of product L^{-1} d^{-1} (KREIS, 1993). LANGE (2018) addressed various considerations for commercial targets like market value/volume, structural complexity/chemical synthesis, regulatory burden and consumer acceptance. He also provided a list of possible new targets, yet avoiding compounds that have 'fallen out of favor among clinicians', and focused on structurally complex and expensive SNAPs not easily accessible from the source plants.

Commercialization is hindered when there is a complex system of players. For example the anti-malaria drug artemisinin was regarded a good target compound and lots of efforts have been made to establish plant cell cultures to study artimisinin biosynthesis and to use them as a source for this valuable compound. So far commercialization of alternative processes, including a very promising yeast process generating semi-synthetic artemisinin, has not been successful (PEPLOW, 2016). This illustrates the complexities of economic forces that affect the market for certain drugs. They also had an adverse impact on the metildigoxin process established in Ernst Reinhard's group in Tübingen. The process was scaled-up to 5 m^3 by Boehringer Mannheim (now Roche) and was regarded economically feasible. But the market for cardiac glycosides deteriorated and the high-yield plants obtained by selection breeding yielded enough digoxin to satisfy the needs in the end.

But what happened to the shikonin process? The demand for shikonin in 1988 was only around 150 kg per year with a market value of 600,000 U.S. $. It turned out that production was not significantly cheaper than the extraction from *Lithospermum erythrorhizon* roots (LANGE, 2018) and hence the process abandoned.

Only recently, a bilberry cell culture process for the production of a polyphenol extract as a dietary supplement was announced by Diana Plant Sciences. They had already launched a cocoa product derived from *Theobroma cacao* L. suspension-cultured cells in 2013 (LANGE, 2018). EIBL et al. (2018) gave an overview of plant cell culture extracts containing SNAPs such as polyphenols, vitamins, fatty acids, peptides for applications in the cosmetics and food industries. Teoside 10, an extract prepared from suspension-cultured *Ajuga reptans* cells was the first plant cell culture extract approved as a food supplement ingredient in Europe. Only time will tell whether this gives notice of a renaissance of plant cell culture or of a sudden hype prone to extinction.

Outlook

Although plant cell culture technologies have been around for decades, it still seems to be difficult to commercialize plant cell culture processes, mainly due to the competition with already existing ways of manufacturing. But without any doubt, plant cell suspension cultures left their mark on various aspects of applied botany related to the elucidation of biochemical pathways and the production of SNAPs. In the genomic and post-genomic era plant cell cultures became less important tools to study biochemical pathways. New technologies emerged and homology search in genome and protein databases allowed for new strategies in pathway elucidation. Even if not all genes and enzymes of a given SNAP pathway are known the SNAP itself may be produced after merging genes from different organisms in an appropriate host. However, each step in a given pathway is subject to uncertainties and there are many cases where the genetic engineering approach did not give the expected results. For example, the tryptophan decarboxylase gene introduced and constitutively expressed in *Catharanthus roseus* (L.) G. Don cells resulted in an increase of tryptamine, but not in an increase of indole alkaloid production (BERLIN and FECKER, 2000). In recent years, microorganisms were genetically engineered with a view to synthesize natural products usually derived from plants. Among these engineered pathways a lot of different target structures can be found, such as phenols, isoprenoids, alkaloids, and polyketides (SIDDIQUI et al., 2012). For example, a *Saccharomyces cerevisiae* strain has been developed, which is capable of synthesizing hydrocortisone from a simple carbon source (DUMAS et al., 2006) and more recently, morphinanes have been produced in *E. coli* (NAKAGAWA et al., 2016). Pathway engineering seems to be a promising approach for the production of a multitude SNAPS. Only recently, we have constructed a yeast expression plasmid containing a cardenolide biosynthetic module. It enables a yeast strain to produce 5β-pregnane-3β,21-diol-20-one, a central intermediate in 5β-cardenolide biosynthesis, starting from pregnenolone which we added to the culture medium. With this approach, five consecutive steps in cardenolide biosynthesis were realized in baker's yeast (RIECK et al., 2019). Two of the enzymes

used, namely Δ^5-3β-hydroxysteroid dehydrogenase and progesterone 5β-reductase were isolated for the first time in the 1980s from *Digitalis* cell and tissue cultures demonstrating again the impact of plant cell culture for basic research and applied botany.

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