Green (cell) factories for advanced production of plant secondary metabolites

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ABSTRACT

For centuries plants have been intensively utilized as reliable sources of food, flavoring, agro-chemical and pharmaceutical ingredients. However, plant natural habitats are being rapidly lost due to climate change and agriculture. Plant biotechnology offers a sustainable method for the bioproduction of plant secondary metabolites using plant \textit{in vitro} systems. The unique structural features of plant-derived secondary metabolites, such as their safety profile, multi-target spectrum and “metabolite likeness,” have led to the establishment of many plant-derived drugs, comprising approximately a quarter of all drugs approved by the Food and Drug Administration and/or European Medicinal Agency. However, there are still many challenges to overcome to enhance the production of these metabolites from plant \textit{in vitro} systems and establish a sustainable large-scale biotechnological process. These challenges are due to the peculiarities of plant cell metabolism, the complexity of plant secondary metabolite pathways, and the correct selection of bioreactor systems and bioprocess optimization. In this review, we present an integrated overview of the possible avenues for enhancing the biosynthesis of high-value marketable molecules produced by plant \textit{in vitro} systems. These include metabolic engineering and CRISPR/Cas9 technology for the regulation of plant metabolism through overexpression/repression of single or multiple structural genes or transcriptional factors. The use of NMR-based metabolomics for monitoring metabolite concentrations and additionally as a tool to study the dynamics of plant cell metabolism and nutritional management is discussed here. Different types of bioreactor systems, their modification and optimal process parameters for the lab- or industrial-scale production of plant secondary metabolites are specified.

Introduction

Plants are notable for their considerable spectrum of chemical products, particularly secondary (or specialized) metabolites (SMs) that possess beneficial biological activities that are important to humans [1]. The integrated utilization of appropriate analytical techniques for natural products (NPs) screening, improved \textit{in vitro} and \textit{in vivo} bioassay models as well as the study of the molecular targets and networking of the bioactive molecules, has led to the isolation of several important anticancer drugs of plant origin, e.g. paclitaxel, etoposide phosphate, topotecan and homoharringtonine [2]. Out of the large number of currently unanalyzed plant NPs, there are likely to be many more such molecules awaiting exploitation [1].

Plant SMs possess several unique features, such as complex and specific arranged aromatic rings, chiral centers, number and the ratio of heteroatoms, which make them not only targets for drug discovery but also a starting point for novel synthetic drugs [3]. Along with the low or absence of toxicity and the broad spectrum of activity, another important advantage of plant-derived drugs is the so-called “metabolite-likeness.” This means that the natural molecules are not only biologically active, but are also the preferred substrates of transporter systems that deliver molecules to their intracellular activity site. For that reason, a quarter of all drugs currently approved by the Food and Drug Administration (FDA) and/or European Medicinal Agency (EMA) are plant-derived. The same transporter systems are also responsible for the outside cell
transport of the drugs, which is a challenge from one side, but also used as an opportunity to study drug resistance mechanisms [4,5].

The intensive utilization of plants and plant-based NPs creates an enormous discrepancy between their demand and availability. Moreover, plant SMs typically present in low amounts and harvesting sufficient materials to supply current needs can be both environmentally destructive and impractical [6]. During the last few decades, there has been an increasing interest in the production of biologically active molecules from plant in vitro systems. They present an alternative perspective for the production of high-value marketable molecules through different biotechnological systems, with major advantages being the consistency of the yield and quality of plant-derived products, shorter production cycles (compared to whole plants), improved biosafety (lack of environmental or genetic contamination) due to controlled aseptic conditions defined by the current good manufacturing practices (cGMP) [7]. The goal of such a bioprocess is to reach high productivity, yield, and the concentration of the desired SMs. A large number of strategies have been used, e.g. design of an appropriate bioreactor system, selection of highly-productive lines, nutrient medium optimization, elicitation, metabolic engineering, two-phase cultivation and plant cell immobilization [8]. Large-scale cultivation has been partially limited by the peculiarities of plant cell structure and their moderate to high shear stress (the critical shear stress is between 50 and 400 N/m²). Therefore, the bioreactor design must ensure a favorable growth environment, sufficient mixing, effective nutrient transport and gas exchange, at low shear stress levels [7].

The productivity of the plant in vitro systems could also be improved by using molecular approaches and nutritional management studies [9]. Metabolic engineering has become a powerful tool for transferring new genes into the plant cells used to enhance a desired metabolic pathway, extending existing routes or introducing new ones to obtain novel compounds [9]. The first conventional action is the identification of the rate limiting steps, followed by overexpression/suppression of the gene(s) regulating them. Therefore, the elucidation of these steps is achieved by multi-omics, co-expression, and integrated network analysis, revealing the correlation between genes, proteins and metabolites [10]. In the era of genome editing, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISSP-associated 9) technology has been used to modify the genes that regulate important biosynthetic pathways in plant in vitro systems aiming to enhance the plant SMs biosynthesis through targeted mutagenesis [11,12]. Metabolomics has been developed as a useful tool for direct chemical insight of the metabolites in heterogeneous samples, and as a fundamental part of the current “omics” technologies [13]. It has many applications in life sciences, including plant biology, pharmacology, toxicology, and nutrition [14] aiming to identify the full metabolite complement (untargeted analysis) or measure selected metabolites (targeted analysis) of a cell, tissue, biofluid, or organism [13]. Analyses on plant in vitro systems, when integrated with “omics” technologies, provide an enormous advantage for the study of cellular processes, gene functions and metabolites network alterations under environmental stimuli. This will result in optimization of the bioprocess parameters during laboratory or large-scale cultivation [15]. A global view of the topics covered in this review is outlined under the integrated scheme presented in Figure 1.

In this review we have attempted to summarize effective approaches to enhance the SMs production in plant in vitro systems through metabolic engineering, based on the regulation of single or multiple genes of the biosynthetic pathways through overexpression of transcriptional factors (TFs). Nuclear magnetic resonance (NMR)-based metabolomics has been discussed as an effective tool for off-line and on-line monitoring of the concentrations and metabolite fluxes during the bioreactor cultivation. Selected examples of small- and large-scale cultivation of plant in vitro systems are presented. The most frequently used bioreactor systems and their modifications, as well as, process parameters optimization have also been considered.

Manipulation of secondary metabolism through metabolic engineering

The possibility of exploiting plant in vitro systems as a “green cell factory” has significantly increased over the last decade. Metabolic engineering through transformation with Agrobacterium species followed by manipulation of plant metabolic signaling pathway has been intensively used to enhance the production of pharmaceutically valuable SMs in plants and plant in vitro systems [16]. The biosynthesis of the SMs is regulated by complex networks. The first-level regulation depends on the structural genes from the investigated biosynthetic pathway, while the second-level regulation is achieved by TFs controlling the expression of the structural genes [17].

Two examples will be discussed here in more detail, namely regulation of the biosynthesis of rosmarinic acid and trans-resveratrol (Figure 2). Rosmarinic acid
(RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which is well-known for its antioxidant and anti-inflammatory potential, the biosynthesis of which has been considered in different plant in vitro systems [15]. The structure of RA incorporates two unsaturated 6-membered rings C1-C6 and C1′-C6′, a double bond at C7′-C8′, an ester group (C9′), a carboxyl group (C9) and four hydroxyl groups (C3, C4, C3′and C4′). It has nine CH groups, one CH2 and eight quaternary carbons. The characteristic feature in the 1H NMR spectrum of RA is the two doublets at ca. δ 7.39 and 6.11 ppm with J = 16.0 Hz assigned as a pair of trans olefinic protons, suggesting that the compound is a trans-caffeoyl ester of 3,4-dihydroxyphenyllactic acid. The aromatic ABX type signals at δ 6.62 (dd, J = 8.0, 1.8), 6.66 (d, J = 8.0), 6.75 (d, J = 8.0), and 7.2 (d, J = 1.8) are assignable of the protons of the 3,4-dihydroxyphenyl unit. The double doublet signals at δ 2.92 (dd, J = 14.3, 9.5), 3.08 (dd, J = 14.3, 3.3), and 5.07 (dd, J = 9.5, 4.0) show the presence of the dihydroxophenyllactic acid unit [15]. The biosynthetic pathways leading to RA accumulation involves the phenylpropanoid and tyrosine-derived pathway, using as precursors the amino acids L-phenylalanine and L-tyrosine [18].

The abiotic elicitor methyl jasmonate has been used to activate the transcripts of PAL and C4H enzymes from the phenylpropanoid pathway, as well as TAT and HPPR from the tyrosine-derived pathway, thus increasing the biosynthesis of RA (from 3.25 to 6.02%) and lithospermic acid B (LAB; from 2.94 to 19.3%) [19]. The cloning of SmC4H and SmTAT genes from Salvia miltiorrhiza in its relevant hairy root (HR) cultures also led to increased amounts of RA [20,21].

Increased research attention has been given to exogenous TFs, such as MYC2, a helix loop helix (bHLH), the up-regulation of which promotes the production of phenolic acids (such as RA and LAB). The RA amount increased 2.46-fold (from 2.59 to 6.36 mg/g), while LAB increased 1.88-fold (from 3.17 to 5.95 mg/g DW). The transcriptome database revealed 21 genes homologous to SmPAL, SmTAT, SmC4H, and SmRAS which were up-regulated. The most essential of them (SmPAL) in the phenylpropanoid pathway was up-regulated 367.1-fold [22]. The expression of Arabidopsis MYB-type Production of Anthocyanin Pigment 1 TF (AtPAP1) up-regulated genes in the main phenylpropanoid pathway (SmPAL1 and Sm4CL), the tyrosine-derived pathway (SmTAT and SmHPPR) and some specific branches leading to the biosynthesis of RA and salvianolic acid B (Sal B), such as RAS. [AQ] The content of Sal B increased 2-fold, reaching up-to 73.27 mg/g DW [23]. Other TFs, such as DEL (Delia, a bHLH-type TF) and ROS1 (Rosea1, a MYB-type TF) controlled by P35S promoter up-regulated the Sm4CL and SmRAS, that correlated with the enhanced biosynthesis of RA and Sal B [24]. However, some MYB-type TFs, such as MYB36 can inhibit the biosynthesis of phenolic acids and flavonoids. The overexpression of...
**SmMYB36** in *S. miltiorrhiza* activated genes from the tanshinone pathway, simultaneously inhibiting those from the phenolic acids pathway [17].

Nicolas et al. [25] investigated the effects on the resveratrol biosynthesis of AREB/ABFs TFs, which contain a basic leucine zipper family (bZIP)-type DNA binding domain and have a pivotal role in ABA-dependent gene activation. The ABA-induced overexpression of VvABF2 TF from grape influenced the expression of 1370 genes containing ABRE motifs in the promoter. The transcripts of some key enzymes from the phenylpropanoid pathway (PAL and C4H), as well as, the resveratrol biosynthesis (stilbene synthase; STS) were considerably increased and resulted in 6.2 and 2.5-fold higher amounts of *trans*-resveratrol and its glycosylated form *trans*-piceid [25]. The overexpression of two R2R3-MYB-type TFs (MYB14 and MYB15), strongly expressed PAL and STS genes in the hairy roots of *Vitis vinifera*, resulting in an increased stilbene production. Both TFs were differently induced when exposed to external stressors,
such as UV-C light, wounding or downy mildew infection. Investigating the differences in MYB14 and MYB15 expression and/or transcriptional activity in different grapevine cultivars might lead to the development of molecular markers for the breeding of grapevines with optimized stilbene content and composition [26]. To increase the amount of trans-resveratrol (up-to 40 μg/L), Hidalgo et al. [16] established a transgenic HRs from tobacco harboring the gene encoding STS from V. vinifera and/or the TF AtMYB12 from Arabidopsis thaliana aiming to activate and coordinate the up-regulation of resveratrol through the phenylpropanoid pathway. Additionally, an artificial microRNA for chalcone synthase (amiRNA CHS) was used to suppress the metabolite flux through CHS inhibition, which might be a competitive pathway that utilize the precursors of the STS enzyme. It was found that after MeJa elicitation, the VvSTS gene expression resulted in increased total stilbene production in the S (contain T-DNA and VvSTS) type cultures, while the co-expression of AtMYB12 TF and VvSTS lead to 9.6-fold higher stilbene production in TF/S (contain T-DNA, VvSTS, and AtMB12 TF) type lines than the S type lines. The gene-to-metabolite network revealed that there was positive correlation between AtMYB 12 TF and the PAL and CHS genes, indicating that the TF can activate early and late genes involved in phenolic acid metabolism. On the other hand in the TF/S/Inh (contain T-DNA, VvSTS, AtMB12 TF, amiRNA-CHS) lines the interference between amiRNA-CHS and the natural CHS gene expression resulted in decreased levels of flavonoids and the lowest levels of stilbene production. This might be related with the simultaneously low expression of VvSTS and AtMYB12 TF genes [16]. Other TFs, such as VvWRKY8 can be used to suppress the resveratrol biosynthesis, e.g. after resveratrol reaches a defined concentration, VvWRKY8 TF binds with another TF, VvMYB14 (which normally increases the resveratrol biosynthesis), and inhibits its binding to the VvSTS 15/21 promoter, resulting in reduction of the resveratrol concentration [27].

The integration of transcriptome and metabolome data are crucial for the study of gene-to-metabolite networks [19]. For example, the PAL transcript was correlated with both RA and LAB, but more closely correlated with LAB. The C4H and HPPR were also related to RA, while TAT is mainly related to LAB biosynthesis and the 4CL enzyme is not correlated to both RA and LAB in statistical sense [19]. Based on this gene-to-metabolite network Xiao et al. [18] performed a more accurate genetic strategy by the selective introduction of single gene constructs containing cDNA clones of TAT, HPPR, C4H, binary TAT and HPPR, as well as, antisense-HPPD genes. It was observed that the transcript levels of C4H, TAT, HPPR and the binary TAT and HPPR were up-regulated by approximately 7.7, 3.0, 6.2, 2.5, and 4.3-fold, while the HPPD transcript was reduced 50%, respectively. The single overexpression of C4H activated the two parallel RA-biosynthetic pathways. An increase in t-cinnamic and 4-coumaric acid from the phenylpropanoid pathway and an increase of L-tyrosine and 4-hydroxyphenylpyruvic acid from the tyrosine-derived pathway lead to high production of RA (211 mg/L) and LAB (584 mg/L). The overexpression of TAT and HPPR separately increased the RA and LAB content up to 616 and 669 mg/L, respectively. However, the highest amount of both phenolic acids was achieved in the double TAT-HPPR gene transformation lines with RA 906 mg/L and LAB 992 mg/L. When this double vector was used the lowest levels of homogentisic acid (a substrate that takes place in a competitive branch to RA biosynthesis pathways) was also observed [18].

CRISPR/Cas9 system: toward genome editing in plant in vitro systems

Clustered regularly interspaced short palindromic repeats (CRISPR) along with CRISPR-associated proteins (Cas) acts as a type of adaptive immunity system in prokaryotes and provides sequence-specific protection against foreign DNA or RNA [28]. In general, the CRISPR/Cas9 technology is based on the location and identification of foreign DNA sequences by small guide RNAs (gRNA) and its cleavage by an associated DNA endonuclease (Cas9). The Cas9 activity is associated with two types of gRNAs, designated as CRISPR RNA (crRNA or the “protospacer”) and trans-acting RNA (tracrRNA) [29]. To enhance the application of this system, especially in eukaryotic cell genome editing, the two separate gRNA sequences are combined into one single guide RNA molecule (sgRNA). The sgRNA loop structure binds to the target sequence and builds a complex with Cas9, which cleaves the double-stranded DNA and forms a DNA double-strand break (DSB) at the target locus, which is further repaired by the host cell repair system. After the DSB is formed, the DNA sequence modification can occur in two ways, nonhomologous end joining (NHEJ) or homology-directed (HDR) [29]. A DSB is usually repaired by NHEJ, which is an error-prone process comprising nucleotide insertions, deletions and substitutions (indels), resulting in gene knockout (inactivation) via a frameshift processes. By contrast, when a DNA template with homologous
arms is present, HDR induces precise specific modification of the genomic sequences [30].

The major application of CRISPR/Cas9 at present is as a genome-editing system, mainly including gene knockouts in organisms elucidating the function of single or multiple gene targets via gene mutation. The CRISPR/Cas9 gene-editing system is also able to generate heritable, targeted mutations in transgene free plants [31].

In order to demonstrate the application of the CRISPR/Cas9 system, a carrot cell culture was used by Klimek-Chodacka et al. [28]. The gene $F3H$, encoding flavonone-3-hydroxylase ($F3H$; EC 1.14.11.9) in the anthocyanin pathway (expressing a purple-colored carrot callus), was blocked by multiplexing CRISPR/Cas9 vectors. This mutation resulted in the growth of white calli, and confirmed the functional role of this gene, as well as, providing a visual marker for screening [28].

The CRISPR/Cas9 targeted mutagenesis has been largely applied to evaluate the function of genes (or gene families) managing important biosynthetic pathways or leading to crop improvement. Some of the main applications of CRISPR/Cas9 are listed on Figure 3. The efficiency of genome modifications is followed by selecting a scorable phenotype expressed in homozygous

![Figure 3](image-url). The mechanism of the CRISPR/Cas9 system for genome editing and its application in plant metabolic engineering. DSB, double-strand break; HDR, homology-directed repair; NHEJ, non-homology end joining; sgRNA, single guide RNA.
CRITICAL REVIEWS IN BIOTECHNOLOGY

The production of plant secondary metabolites in bioreactors

The scaling-up of plant in vitro systems cultivation into large-scale bioreactors is the final step of a bioprocess for continuous and sustainable production of low-volume and high-value bioactive molecules. Economically feasible bioreactor cultivation must guarantee high SMs productivity, yield and concentration by selecting optimal bioreactor design and operating conditions [36], thus ensuring adequate mixing (facilitate nutrient transfer, prevent accumulation of toxic metabolites and simultaneously maintain a low shear stress environment), gas exchange (deliver sufficient O2 or CO2 for respiration) and homogeneity (prevent cell sedimentation) [37].

Bioreactor production of SMs also depends on the pharmacological significance and health benefits for humans of the target SMs. Selected examples are the production of RA, and resveratrol, and an overview is presented in Table 1. Both molecules are characterized with multiple health benefits during the treatment of neurodegenerative disease [42]. Many RA mutants. Such investigations have been performed in many important vegetable cultures and crops or their relevant in vitro systems, which are further used to recover transgenic plants. For example, several root-specific studies have been performed using plant in vitro systems. The symbiosis receptor-like kinase (SYMRK) is essential in the formation of nodules and for the symbioses of legumes with rhizobia, while the leghemoglobin genes are important for the oxygen transfer and energy status within nodules for efficient symbiotic nitrogen fixation (SNF). The expression of SYMRK in wild type plants formed pink nodules (necessary for SNF) even in the absence of rhizobia. However, the simultaneous mutagenesis toward all these genes resulted in the formation of transgenic plants with white nodules (a nitrogen deficiency phenotype) [32]. The expression of the GmMYB118 gene increased the drought and salt tolerance of soybean plants, while CRISPR-transformed plants had reduced tolerance, accompanied by higher levels of reactive oxygen species (ROS) and malondialdehyde (a marker of oxidative stress) [11]. Investigations with knockout mutants revealed the role of broad spectrum resistance 1 (BSR1) genes in the defense system of rice toward the blast fungus [33], while the mutations in the VvWRKY52 gene from the WRKY TF family revealed its role in the resistance of grape toward Botrytis cinerea that causes rot on the grape’s fruits [34].

CRISPR/CAS9 technology has been used to manipulate plant secondary metabolism as well as the production of recombinant proteins. Nowadays, with respect to secondary metabolism, the CRISPR/CAS9 technology is mainly used to investigate the biosynthetic potential of plant in vitro systems through switching off of the competing biosynthetic pathways and eventual shifting of the metabolite flux toward the production of the target compounds. The RAS gene knocked-out homozygous mutants of S. miltiorrhiza transgenic hairy roots resulted in significantly decreased levels of RA and LAB, while the heterozygous lines generated only a slight reduction [31]. The knocking out of SmCPS1 gene in S. miltiorrhiza blocked the metabolic flux through geranylgeranyl diphosphate (GGPP) and disrupted tanshinone biosynthesis. Since tanshinones and taxol have the same precursor (GGPP), the authors consider that, in theory, GGPP could serve as a source for other valuable diterpene biosyntheses, such as taxol [35]. The production of recombinant proteins in plants or plant in vitro systems has several advantages compared to their biosynthesis in microbial and mammalian cultures, such as complex protein folding and post-translational modifications. However, the plant produced proteins carry N-glycans with typical residues [β(1,2)-xylose and core α(1,3)-fucose], which can induce an immune or allergic response in humans. This glycosylation is carried out by the enzymes β(1,2)-xylosyltransferase (XyIT; EC 2.4.2.38) and α(1,3)-fucosyltransferase (FucT; EC 2.4.1.65). In order to produce IgG2 antibody without any β(1,2)-xylose or α(1,3)-fucose residues from N. tabacum BY-2 cells, the XyIT and FucT genes were knocked out by targeting conserved regions with CRISPR/Cas9. The absence of the glycans in the produced IgG2 antibody revealed that it can be engineered to humanize pharmacological glycoproteins [12].

Despite the great progress of CRISPR/Cas9 genome editing system, there are still many gaps to fill about its off-target effects in plant genome and how to eliminate it and the effectiveness of the transmission rate of the mutation onto the next generations. The mechanism of dissociation of Cas9 from designed sgRNA and its subsequent recycling is still unknown and requires further research. Besides all of these questions, the CRISPR system has many advantages over traditional genetic engineering methods. Since CRISPR/Cas9 utilizes ribonucleoprotein complexes for creating DSB, their design is faster, easier, more precise, cost effective and allows for the editing of multiple target genes simultaneously. Simple redesign of sgRNA to change target specificity in CRISPR/Cas9 is only required, whereas in ZFNs and TALENs a new DNA-binding protein has to be synthesized for every gene target. In addition to the listed advantages, the CRISPR/Cas9 system is more efficient at targeted genome editing than other nucleases [31].
biotechnological platforms, including suspension, shoots or hairy root cultures [38,40] from different plants, such as Ocimum basilicum [45], Salvia officinalis [40], Coleus blumei [38], and Dracocephalum forrestii [39] have been developed. Different types of bioreactors and strategies to enhance the RA accumulation, such as metabolic engineering and elicitation with abiotic/biotic elicitors have been applied [18]. Nowadays, plant cell suspension culture technology has paved the way to be the most convenient plant in vitro system for the biosynthesis of SMs at lab- or large-scale level due to its homogenous growth pattern, shorter production cycles and more simplified bioreactor construction. A low shear stress environment in a stirred tank bioreactor (equipped with one marine impeller operating at 100 rpm) allowed the accumulation of 30 mg/g DW RA during the cultivation of an O. basilicum cell suspension [45]. However, as dedifferentiated cultures, cell suspensions remain heterogeneous over time resulting in poor growth and a low/inconsistent yield of NPs due to deleterious genetic and epigenetic changes. For that reason, shoot or hairy root cultures, as differentiated plant tissues, such as elicitors. Only the “bioreactor-elicitation” cultivation regime (200 μM MeJa) was able to achieve the maximum RA production by the O. basilicum suspension culture in a stirred tank bioreactor. The accumulated RA was 2.56 times higher than in the non-elicited culture [45]. The addition of 100 μM MeJa increased the RA production in Satureja khuzistanica Jamzad cell suspensions more than 3-fold (3.9 g/L) without affecting biomass accumulation in flasks. Further, the accumulation of 3.1 g/L RA, due to the elicitor, confirmed its sustainable production in a wave-mixed bioreactor [47].

The relative recent isolation of the cambial meristematic cells (CMECs) provides an emerging platform for the encompassing of the potential difficulties with the plant cell suspensions. Unlike the plant cell

### Table 1. Production of rosmarinic acid and resveratrol in different types of bioreactors.

| Plant species                  | Culture type and metabolite | Bioreactor type and volume | Bioreactor operating conditions                                                                 | Metabolite content, mg/L | Metabolite content, mg/g DW | Reference |
|-------------------------------|-----------------------------|---------------------------|-----------------------------------------------------------------------------------------------|--------------------------|-----------------------------|-----------|
| **Rosmarinic acid**           |                             |                           |                                                                                               |                          |                             |           |
| *Coleus blumei* L             | Hairy roots                 | 1-L airlift               | 24°C in dark; 0.5 L/s flow rate                                                                | 871.80                   | 35.0                        | [38]      |
| *Dracocephalum forrestii W. W. Smith* |                       | 10-L nutrient sprinkle    | 26°C; 16 h/8 h light/dark regime; 25 s pump operating time/2.5 s breaks                       | 38.26                    | 17.90                       | [39]      |
| *Salvia officinalis* L        | Hairy roots                 | 5-L nutrient sprinkle     | 26°C in dark; 40 s pump operating time/50 s breaks                                             | 477.13                   | 34.65                       | [40]      |
|                               | Shoot cultures              | 26°C; 16 h/8 h light/dark regime; 45 s pump operating time/40 s breaks |                                                                | 59.04                    | 26.24                       |           |
| **Resveratrol**               |                             |                           |                                                                                               |                          |                             |           |
| *V. amurensis* Rupr            | Cell suspension             | 3-L balloon type airlift  | 25°C in dark; 0.2 vvm L/min flow rate                                                           | 53.80                    | 0.15                        | [41]      |
| *Vitis labrusca* L            | Cell suspension             | 5-L stirred tank          | 23°C in dark; 100 rpm agitation; 0.025 vvm flow rate                                             | 66                       | 6.53                        | [42]      |
| *V. labrusca* L               | Cell suspension             | 14-L stirred tank         | 23°C in dark; 50 rpm agitation; 0.025 vvm flow rate                                             | 72                       | 1.44                        | [43]      |
| *V. vinifera cv.* Chasselas × Vitis berlandieri | Cell suspension | 2-L stirred tank          | 23°C in dark; 50 rpm agitation; 0.025 vvm flow rate                                             | 209                      | 1.40                        | [44]      |
suspensions, which are dedifferentiated cell cultures, the CMCs are undifferentiated cells that grow indefinitely and function as plant stem cells, since they are isolated from procambium meristematic cells, and only consist of true meristem stem cells, which are responsible for the production of xylem and phloem within the vasculature [48]. In this way, the CMCs circumvent the dedifferentiated procedure [49], omit the epigenetic modifications and are characterized with fast and uniform growth as single cells, possess morphologically and physiologically stability and are easy to regrow after cryopreservation [50,51]. The reduced aggregation of CMCs prevents cluster formations, thus overcoming the oxygen and nutrient deficiency for the cells and the low product yield, frequently observed in the dedifferentiated cells [48]. Another advantage of CMCs is their high responsiveness to elicitation. For instance, treatment of C. roseus CMCs with 10 mM β-cyclodextrin and 150 µM MeJa or combination of both of them resulted in 32, 15 and 108% increase of ajmalicine production, compared to the non-treated cells [52]. Elicitation with 100 µM MeJa increased the production of paclitaxel in T. cuspidata CMCs by 14,000%, while the production of paclitaxel within needle- or embryo-derived dedifferentiated cells was 220 and 430% respectively [49]. The advantages of CMCs have been employed by the Korean company “Unhwa Corp.” that possesses the world’s first patent technology for CMC isolation and cultivation named Ddoyul™. This technology has been applied for the development of CMC-based suspension cultures of T. cuspidata, Ginkgo biloba and Solanum lycopersicum with application in the cosmetic industry [48].

Resveratrol (3,4′,5-trihydroxystilbene) is the most valuable biologically active stilbene and has been biosynthesized from plant in vitro systems of several taxonomically unrelated species, such as Polygonum cuspidatum, V. vinifera, Vitis rotundifolia, and Arachis hypogaea [53]. The bioreactor cultivation of resveratrol requires optimization of the culture medium, agitation and aeration rate, during batch cultures in stirred tank bioreactors. The biosynthesis of resveratrol by Scrophularia striata cell suspensions in a 10-L stirred tank bioreactor was approximately 60 times higher than that in shake flasks by optimization of the aeration and agitation speed of the bioreactor. The cultivation started at 0.5 L/min aeration and agitation of 110 rpm, which increased up to 1.5 L/min and 170 rpm with an enhanced cell density [54]. In a similar way, aeration during the cultivation of a cell suspension of V. vinifera, increased from 0.075vvm on the first day of cultivation to 0.15 vvm at the fifth day, while maintaining the same agitation speed [55]. The use of 0.2 mM MeJa was the only possibility for the biosynthesis of resveratrol by a cell suspension of V. vinifera cv. Chasselas × Vitis berlandieri in a 2-L stirred tank bioreactor. About 90% of the resveratrol (209 mg/L) was found in the cultural medium, only 21 mg/L in the cells, while no resveratrol was produced by the control culture. Methyl jasmonate could eventually induce the biosynthesis of some resveratrol derivatives (ε-viniferin, δ-viniferin, 3-methylviniferin, and piceatannol), which were reported to be produced for the first time in a bioreactor [44]. The addition of 13 mM cyclodextrines during the cultivation of a V. labrusca cell suspension in a 14-L stirred tank bioreactor resulted not only in production of resveratrol, but also of ε-viniferin, pallidol, and labruscol [43,56]. Fed-batch processes improve the production of resveratrol in comparison to batch cultivation. The kinetics of resveratrol production was evaluated by monitoring biomass, sugars, resveratrol concentration, and dissolved oxygen levels during a fed-batch cultivation of V. labrusca in a 5-L stirred tank bioreactor. Fresh medium was added at 141 and 212 h respectively, while the elicitor (0.5 mM MeJa) was added when sucrose was completely depleted. The resveratrol concentration remained approximately zero during the first 7 days after elicitation and reached a maximum of 66 mg/L on day 12 [42]. After the optimization of the sucrose (30 g/L), inoculum (40 g/L) and chitosan (50 mg/L) concentrations in a batch process, a fed-batch process in 1-L stirred tank bioreactor for the cultivation of V. vinifera cv. Barbera was performed, during which the resveratrol concentrations increased 74 fold [53]. Optimization of several parameters (20 g/L inoculum, 30 g/L sucrose and 500 mg/L casein) during V. amurensis cell suspension culture in a 3-L balloon type bioreactor resulted in the production of 53.80 mg/L resveratrol [41].

Plant in vitro systems have been recognized as a reliable source of pharmaceutically relevant SMs. However, the success of large-scale bioreactor cultivation depends on the culture conditions and the bioreactor design, the choice of which is in accordance to the NPs to be biosynthesized, the culture type used and can even vary between the different lines from the same in vitro culture. Stirred tank bioreactors and fed-batch processes combined with metabolic engineering have been the most suitable for the cultivation of plant cell suspension cultures, while the cultivation of HRs or shoot cultures requires the use of bioreactors associated with less shear stress, such as airlifts or nutrient sprinkle bioreactors. Parameters such as mixing and aeration need to be optimized in order to obtain optimal cell growth and product formation.
Metabolomics for high-resolution monitoring of secondary metabolism and cell growth in bioreactors

Metabolomics is a powerful and comprehensive analytical platform for the global identification and quantification of all metabolites (primary and secondary) in a cell, tissue or organism [14,57]. Along with other complementary “omics” technologies (transcriptomics and proteomics), metabolomics has evolved as a tool in the functional annotation of genes, offering a broad view of the biochemical status of an organism, used to monitor significant metabolite variations due to genetic or environmental changes [14,58].

Metabolomic studies are a sought application of non-targeted (metabolite fingerprinting) or targeted (metabolite profiling) analyses [59], thus developing genotypic (single-nucleotide polymorphism) and phenotypic (at transcript, protein or metabolite levels) biomarkers, determining the extensive application of metabolomics in plant biology, food science, pharmacology, and medicine [60]. In plant science, metabolomics has been applied to study metabolite differences between plant species or between their different organs [61], chemotaxonomic classification [62], distinguish the geographical origin of plants [60], drug discovery, quality assessment of plant-derived products [63], study the metabolic alterations in genetically modified plants or plants subjected to specific environmental stimuli [60]. NMR-based metabolomics has several advantages compared to mass spectrometry (MS) coupled to separation techniques such as gas chromatography (GC)/MS, liquid chromatography (LC)/MS and ultra-high performance liquid chromatography-mass spectrometry (UHPLC/MS). It is a rapid, nondestructive and nonselective platform for the simultaneous detection of primary and secondary metabolites in complex plant extracts without the need of prior chromatographic separation or derivatization of the analytes. In addition, via NMR analysis it is possible to obtain quantitative information, since the signals of the metabolites are proportional to their molar concentration. Major limitations of NMR spectroscopy are the lower sensitivity (micromolar range) in comparison to MS (picomolar range) and the overlapping signals in the NMR spectrum [64]. The sensitivity could be improved by using high field magnets, cryogenic probes or microcoil probe heads [57,58]. The signal overlapping in one-dimensional (1D) 1H NMR hampers the metabolite identification, but this could be solved by applying two-dimensional (2D) NMR that has better resolution [64]. Other analytical techniques that deserve to be mentioned are the Fourier transform-infrared (FT-IR) and near-infrared spectroscopy (NIR). They are both nondestructive methods commonly used for the fingerprinting of different natural matrices, especially the quality control of medicinal plants and for rapid qualitative evaluation of sample preparation during metabolomics. These techniques measure the absorption of infrared radiation by the sample material versus wavelength. The infrared absorption bands identify molecular components and structures by ascertaining the concentration of key functional groups such as ROH, -SH, C = C, -CH, -OH, and -NH groups [57,63].

Advances in plant in vitro systems research have enabled the commercial production of important plant secondary metabolites which exhibit health promoting properties for humans. This section aims to focus on the applicability of NMR-based metabolomics for monitoring SMs production in plant in vitro systems during batch, feed-batch or continuous cultivation in flasks and bioreactors. Understanding plant cell metabolism and its regulation as well as the physiological development of the cells are of critical importance for the bioreactor cultivation of plant in vitro systems [65].

NMR-based metabolomics has been applied to trace alterations in primary and secondary metabolites between several Verbascum species and their respective HRs. Verbascoside, forsythoside B, leucosceptoside B, and martynoside were identified in both V. xanthophoe nicum plants and their relevant HRs [66]. The V. nigrum HRs did not produce chlorogenic acid, harpagide and harpagoside, which were identified in the mother plant [67]. In V. eriophorum amongst the secondary metabolites, were identified only verbascoside and martynoside and its amount was in a higher concentration than in the intact plant. Due to structural similarities, the use of TOCSY spectra was essential in the structural identification of verbascoside and martynoside [68]. In the HR lines of V. nigrum significant accumulation of the amino acid glutamine was observed, which was hypothesized to be due to not only to the random integration of the A. rhizogenes T-DNA in the plant genome, but also to the increased need for nitrogen during hairy root growth [67]. The TOCSY provides information such as the unbroken chains of coupled protons in the same molecule. It illustrates a correlation between all protons that make up a spin system. In a TOCSY spectrum the different structures produce independent cross peaks according to their respective J-coupling networks. For each species, the intensities of the cross peaks are determined by the J-coupling constants in the proton coupling network, which are characteristic for the structures and the TOCSY mixing time. Therefore, the intensities of the cross peaks are proportional to the structures themselves and...
During the applied culture conditions, the production of the isotopically labeled L-phenylalanine. Select optimal process parameters and study the metabolomics to measure the cell growth and the metabolite concentrations after the end of the biotechnological process. However, the metabolite concentrations only illustrate the current “state” of a metabolic system. It is commonly accepted that plant cells have a complex metabolic capacity to adapt toward the changing environment, which could be accurately analyzed by metabolic flux analysis (MFA) and metabolic control analysis (MCA). The flux analyses represents what the metabolism is “performing”. The fluxes and turnover rates indicate cell growth, the primary and the secondary metabolism biosynthetic potential. Therefore, it is essential to enable comprehensive characterization of the physiological state of the cell cultures at different stages of the cultivation process through MFA of the intracellular and extracellular metabolic state and predict the behavior of the cultivation system. Metabolomics deliver the closest insight into a physiological process, therefore the integrated analysis of large metabome data is necessary for accurate modeling of the metabolism and to determine the factors leading to the observed or possible physiological state of the cell culture. In most cases the central primary metabolism, which includes glycolysis, pentose-phosphate pathway (PPP), TCA cycle and the analysis of some primary metabolites, such as amino acids, organic acids, organic phosphates, lipids, and structural hexoses have been investigated. Some nutrients, such as intracellular inorganic phosphate (Pi), have gained a special focus since it plays a central role in the regulation of enzyme activities through phosphorylation/dephosphorylation processes, energetic...
shuttles concentrations and equilibrium (ATP/ADP concentration ratio) and the flux distribution between glycolysis and PPP [9,78]. Therefore, MFA is useful to understand the significance of nutritional management and reveal that the stabilized intracellular reserves (e.g. P, nitrogen and sugars) are more important critical factors for secondary metabolite production than traditional medium optimization. The data describing the physiological, nutritional, metabolic states and dynamic behavior of the cell population could be used for further scaling-up of a bioreactor process [72]. It is also possible to identify metabolic sub-networks and discriminate metabolite pools in specific cell compartments [79], which could be useful to understand and predict the regulation of some secondary metabolites, such as TIA, in which biosynthesis occurs in different subcellular compartments [70]. In the heterotrophic Arabidopsis cell suspension, the redistribution of the central metabolism fluxes was followed up through labeling experiments with [1-13C]-, [2-13C]-, and [U-13C6]-glucose. Focusing on the PPP it was established that the oxidative steps from this pathway were duplicated in the cytosol and plastids, with flux through these reactions occurring largely in the cytosol [79]. An example of NMR spectroscopy used as a real-time monitoring tool of physiological parameters, including cell viability and growth during the cultivation of plant cell suspensions, is described by Maish et al. [80]. The authors have developed a microfluidic chip for measuring different metabolites (sucrose, fructose, glucose and myo-inositol) in the flow-through that correspond to the growth state of the tobacco BY-2 cell suspension and the metabolic activity of the cell suspension. For example, the levels of glucose and myo-inositol were very low during the first four days of the cultivation, which corresponded to intensive proliferation, while after day five till the end of the process the levels of glucose and myo-inositol increased due to the decreased catabolic activity and proliferation phase [80].

The dynamic investigations of MFA have found further applications to assess and control some important processes that occur during bioreactor cultivation, which severely hampers the large-scale cultivation of plant in vitro systems, e.g. the study of biotransformation reactions (incl. detoxification) of some SMs, evaluation of the cytotoxic potential of drugs and monitoring the dynamic change in SMs biosynthesis. Han et al. [81] combined the advantages of ultra-performance liquid chromatography/quadruple time-of-flight mass spectrometry (UPLC/Q-TOF/MS) in terms of high peak capacity, high resolution, sensitivity and accurate mass measurement in order to study the alterations of taxoid metabolism in T. chinensis var. mairei cell suspension in response to shear stress. This procedure identified 49 metabolites, 21 of which were taxoids that were further categorized into 8 classes. Unsupervised principal component analysis (PCA) revealed the differences in the metabolite content between the stress-induced and control cells. The shear stress led to a significant increase of 10-deacetyllyunnanaxane, while a decrease in 9-dihydro-13-acetylbacatin III, 5-deacetyltaxachitriene B, 10-hydroxyacetylbaccatin VI was observed [81]. Chromatographic separation and an HPLC-ESI-MS system has been applied to explain the browning phenomena that occurred during the bioreactor cultivation of T. chinensis and Glycyrrhiza inflata cell suspension by the development of transcriptional and metabolite profiles of browning compounds. It was found that browning occurs during the first three days of bioreactor cultivation when enhanced extracellular phenolic accumulation, polyphenol oxidase and sucrose were observed. The identified flavonoids (myricetin, daidzen, quercetin, naringenin, genistein, and kaempferol) by HPLC-MS were regulated by sugar metabolism, such as sucrose. Therefore, by means of metabolomics was developed not only a model elucidating the enzymatic browning mechanisms, but also effective control approaches over it. The inhibition of some genes encoding enzymes involved in sucrose metabolism and the phenylpropanoid pathway by the addition of gibberellic acid (GA3) resulted in effective control of the enzymatic browning of both suspensions [82]. The perfusion NMR system has recently been used to study, in real time, the reaction of glycosylation scopolin to scopolin, which has improved the anti-inflammatory properties. Because of their structural similarities between the two metabolites 2 D TOCSY and NOESY were used along with the 1H NMR [83]. This system has evolved even during the evaluation of the cytotoxic potential of pharmacological drugs. For example, the cytotoxic effect of the drugs calyx-NH2 and 5-fluorouracil was evaluated on a human cervical (HeLa) cancer cell line cultivated in an NMR perfusion bioreactor by monitoring the 31P signals that correspond to intracellular high-energy phosphorous compounds [84]. Nuclear magnetic resonance-based metabolomics along with UPLC-MS/MS analysis was used to study the enzymatic detoxification of dehydroabietic acid (DHA), which is an abundant wood resin and is often found in waste water. The coupling of these two techniques allowed following not only the biotransformation of the dehydroabietic acid but also the concomitant alteration during cell metabolism. The cell suspension and HRs of
N. tabacum converted the DHA into a DHA-18-O-glucoside intracellularly, since the gradual disappearance of DHA from the medium was observed. However, the presence of this substance actually acted as an elicitor, since elevated amounts of alanine, GABA and valine were measured in the cell suspension, whereas some alkaloids, e.g. nicotine, anatable, and anatabine were observed to increase in the HRs. The cell suspension and hairy roots of C. roseus converted DHA first into a hydroxylated derivative and later to a glucosylated one. The structure of these intermediates was confirmed by 1D and 2D NMR spectra as well as Orbitrap-MS analysis, confirming the monohexose conjugates of DHA [85].

Conclusions and future perspectives

Enhanced knowledge of the complex plant metabolic networks involved in the biosynthesis of important biologically active metabolites and a deeper understanding of the regulatory system that governs secondary metabolism could potentially allow successful metabolic engineering of desired molecules in plant in vitro systems. Integration of omics technologies – genomics, proteomics, transcriptomics and metabolomics is important to understand the plant biosynthetic pathways leading to SMs production and ultimately in determining how to manipulate those pathways. Crucially, the implementation of transcriptome and metabolome data is required to study gene-to-metabolite networks for secondary metabolism in plants at both a regulatory and catalytic level. This would be useful in revealing the close relationship between genes and their targeted compounds, resulting in the identification of possible gene candidates linked to their biosynthesis. CRISPR/Cas9 has been successfully used for genome engineering in many plant in vitro systems with the aim to enhance the plant secondary metabolism or identify important genes responsible for the improved resistance toward different biotic or abiotic factors. Thus, it is possible to select the desired phenotypes of crops or medicinal plants at a very early stage, even at the callus level.

Critical for obtaining high yields of the desired SMs in bioreactors is the application of different optimization procedures on process parameters, medium optimization, precursor feeding or elicitation. The selection of appropriate bioreactor configuration is a complex task. Although there are a large number of reusable or disposable bioreactors available, the ideal choice depends on both types of culture and the targeted product scale. The cultivation parameters (especially mixing and aeration) must be precisely equilibrated to achieve optimal growth and product synthesis. Up to now, most of the small- or large-scale cultivations have been performed in stirred tank bioreactors, since they ensure optimal homogenous mixing and keep the shear forces at tolerable levels.

Various challenges are available during the scaling-up of the biotechnological process due to the particular nature of plant in vitro systems and their metabolism are possible. A reasonable solution to both, study and control metabolite concentrations and metabolite fluxes during bioreactor cultivation could be performed by off-line and on-line (in vivo) NMR-based measurements. Such in vivo NMR experiments have revealed their potential to be used for direct characterization of the physiological and metabolic state of living cells or tissues in a noninvasive way, and follow the metabolic changes in response to environmental modifications. Dynamic metabolic fluxes can explain cell behavior during submerged cultivation in terms of cell viability, division and growth as well as describe the kinetics of primary and secondary metabolic pathways and their regulation via intracellular nutrient levels. The development of efficient modeling strategies for energetic management in plant cells, and the modulation of plant metabolic pathways leading to target metabolite production by optimization of growth conditions, could help to improve the production of important bioactive molecules through the use of bioreactors.

Acknowledgements

The authors are grateful to Dr. Rafe Lyall, a Postdoctoral Researcher at the Department of Bioinformatics and Mathematical Modeling of the CPSBB, for proofreading and linguistic editing of the manuscript.

Disclosure statement

The authors report no conflict of interest.

Funding

This project for establishment of CPSBB has received funding from The European Union’s Horizon 2020 research and innovation programme under grant agreement No PlantaSYST – SGA/CSA: 739582 – under FPA: 664620.

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