Isoprenoid Production via Plant Cell Cultures: Biosynthesis, Accumulation and Scaling-Up to Bioreactors

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Chapter 23
Isoprenoid Production *via* Plant Cell Cultures: Biosynthesis, Accumulation and Scaling-Up to Bioreactors

Alexander M. Nosov, Elena V. Popova, and Dmitry V. Kochkin

Abstract Plant cell culture is traditionally viewed as a unique artificially created biological system representing a heterogenous population of dedifferentiated cells. This system undergoes a continuous process of autoselection based on the intensity and stability of cell proliferation. We discuss here the details of formation and regulation of isoprenoid biosynthesis in plant cell *in vitro* based on literature survey and our research. Obviously, secondary metabolism differs in cell culture compared to the plant *per se*, because in cell culture metabolites are synthesized and compartmentalized within a single heterotrophic cell with sparse or underdeveloped vacuoles and plastids. For example, in plant cell cultures isoprenoid biosynthesis *via* MVA pathway was found to be more active than *via* plastid-localized MEP pathway. Also, it was hypothesized that cell cultures preferably produce metabolites, which promote cell proliferation and growth. Indeed, cell cultures of *Dioscorea deltoidea* produced mainly furostanol glycosides, which promoted cell division. Triterpene glycosides (ginsenosides) in the cell cultures of various *Panax* species are represented mainly by Rg- and Rb-groups. Rb ginsenosides are predominantly found as malonyl-esters that may influence their intracellular localization.

Despite the difference in the isoprenoid composition in plant and cell culture the latter became an attractive source of phytochemicals as an alternative to plant harvesting. We provide in this chapter the guidelines to biotechnological production of plant isoprenoids using plant cell cultures and discuss the optimal methods of bioreactor-based cultivation and cryopreservation of plant cell collections.

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23.1 Introduction

Secondary metabolism plays an important role in plant's life. Out of complete genome sequence 15–25 % genes encode enzymes and protein factors involved in biosynthesis of a broad range of so called "secondary products" [1]. These include over 40,000 isoprenoids – or terpenoids, – that play diverse physiological roles in both primary and secondary metabolism [2].

Photosynthesis, respiration, cell organization, compartmentalization and ontogenesis are the common examples of isoprenoid action in primary metabolism [3]. Indeed, carotenoid pigments serve as effective light harvesters and anti-oxidation agents [4]. Sterols are essential components of cell membranes modulating its properties [5]. Side chains of chlorophylls, ubi- and plastoquinones are derived from terpenoids. Phytohormones such as gibberellins, brassinosteroids, abscisic acid and strigolactones as well as cytokinin side chains are of isoprenoid origin [1, 3].

As secondary metabolites isoprenoids are involved in plant – environment interaction including defense against biotic and abiotic stresses, attraction of pollinators etc., suggesting that their physiological functions are even more diverse and complicated [6]. Isoprenoids are the active components in many medicinal plants that have been used for centuries in traditional medicine all over the world. At present they attract commercial interest as potential pharmaceuticals and nutraceuticals [1, 7]. In this chapter, we review in detail the major differences in isoprenoid composition and biosynthesis in *in vitro* plant cell cultures when compared to intact plants and discuss recent advances and challenges of their biotechnological production in bioreactors for pharmacological use.

23.1.1 Introduction to Isoprenoids

Monoterpenes and sesquiterpenes ($\mathbf{C_{10}}$ and $\mathbf{C_{15}}$) is a large family of organic molecules of either 10 or 15 carbon atoms respectively. They are divided into two classes: aliphatic terpenes such as citral from lemons and cyclic terpenes with one or two carbon rings such as menthol, carvacrol, and camphor. In nature mono- and sesquiterpenes are found as highly volatile and strongly scented liquids. They contribute to the scent, flavor and color of plant essential oils and often show pharmaceutical activity. For example, a sub-group called sesquiterpene lactones includes compounds with a bitter taste and is currently being tested on cardiomodulating, anti-bacterial (aucubin) and anti-cancer (arglabin) activities [1, 3].

Diterpenoids **(C_{20})** are the staple components of plant resins from pine, spruce, fir and cedar. Diterpenoids can be classified as linear, bicyclic, tricyclic and tetracyclic diterpenoids depending on their carbon skeletal core. Resin acids, or diterpenoid acids, have the same structure being composed of three hexacarbon rings. Diterpenoids of *ent*-kaurene type have skeleton with four rings. Some diterpenoids have a unique structure such as paclitaxel from yew (*Taxus spp*.) [1, 3]. Diterpenoids have been known for centuries for their antimicrobial qualities. Paclitaxel, or taxol, has become one of the major cytostatic anticancer agents of plant origin. Steviol glycosides produced in *Stevia* leaves is up to 300 times sweeter than sucrose and is extensively used to replace sugar in the diet of patients with diabetes [8].

Triterpenoids **(C_{30})** are derivatives of triterpene molecules and are divided into the following groups:

(i) *Steroidal glycosides (SG)* are glycosides based on C27 steroid-type aglycones with a modifiable side chain, transformed into one (furostanol glycosides) or two (spirostanol glycosides) heterocyclic rings. SG are wide-spread: they have been found at least in 15 plant families including Dioscoreaceae, Liliaceae, Solanaceae, Leguminosae, Costaceae and others. These substances have a broad spectrum of pharmacological activities from anticancer to immune-modulating and sex-stimulating. It is important to note that furostanol and spirostanol type glycosides often show different, even opposite pharmacological activities [9].

(ii) *Triterpene glycosides (TG)* are found in over 30 higher plant families and contribute to unique pharmacological activities of ginseng, aralia, astragalus and glycyrrhiza. Similar to steroid glycosides, TGs are classified based on carbon skeleton of their aglycons. Ginseng glycosides (ginsenosides) found exclusively in *Panax* species are derivatives of two types of tetracyclic aglycons: protopanaxadiols and protopanaxatriols. Pentacyclic compounds are represented by derivatives of ursan, oleanan, lupan and gopan. Glycosides of oleanolic and ursolic acids contribute to biological activities of glycyrrhiza and polyscias [10, 11].

(iii) *Cardiac glycosides* are detected in 13 plant families. Glycoside producing plants include digitalis, lily-of-the-valley, adonis and strofant. Over 400 cardiotonic glycosides have been identified so far. Most common are cardenolides and bufadienolides with additional butenolide or pentadienolide rings, respectively. Cardiac glycosides demonstrate strong heart-beat-modulating and heart-stimulating activity and are irreplaceable with any available synthetic medicines [12].

(iv) *Phytoecdysteroids* are polyhydroxylated steroids found in over 400 plant species from Compositae, Caryophyllaceae and Labiatae families. High phytoecdysteroid content, over 1 % dry weight, was reported in *Serratula spp., Ajuga spp., Rhapontucum spp.* Their environmental function is to protect the plants against insect attack. In humans phytoecdysteroids show well-documented adaptogenic, psychoactive drugs and anti-cancer activities [13].

Tetraterpenoids (C40) in plants are represented mainly by carotenoids: carotenes and xanthophylls. While only 20–30 tetraterpenoids play a role in primary metabolism of the vast majority – over 700 compounds, – are involved in secondary metabolism. They generally function as lipophilic pigments localized in plastids, mainly chromoplasts. Carotenoids demonstrated high and various physiological activities in humans. For example, lycopene and lutein have been recently registered as oncopreventive agents [4].

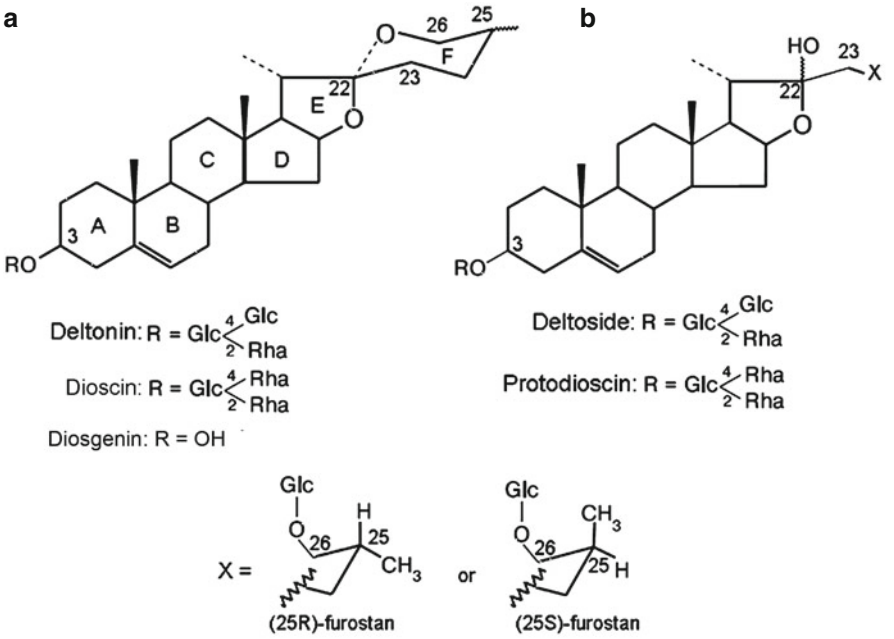
The chemical structures of tri- and diterpenoids discussed in this chapter are shown in Figs. 23.1 and 23.2 respectively.

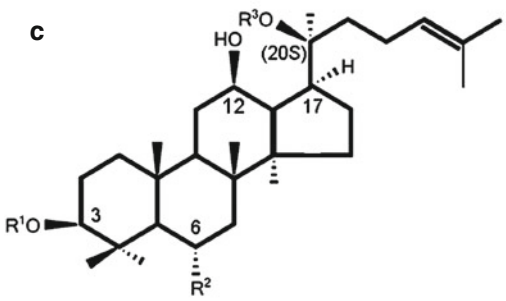
23.1.2 Brief Overview of the Isoprenoid Biosynthesis in Plants

All diversity of isoprenoid structures arises from two isomeric five-carbon (C5) precursors – dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) [16]. Assembly of two, three or four C5 units by prenyl transferases (PT) yields geranyl diphosphate (GDP; C10), farnesyl diphosphate (FDP; C15) and geranylgeranyl diphosphate (GGDP; C20), respectively [17]. Pairwise condensation of FDPs or GGDPs produces squalene (C30) or phytoene (C40), respectively. GDP, FDP, GGDP, squalene and phytoene are the substrates for a large family of terpenoid synthases (TPS) [18, 19], and the immediate precursors of all monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids, respectively. TPS catalyze enzyme-specific isomerizations, various rearrangements and cyclizations yielding the vast pool of cyclic and acyclic terpenoid carbon skeletons found in plants. Many plant TPSs are promiscuous, forming multiple products from a single substrate [20, 21]. Subsequent modifications of the basic parent skeletons synthesized by TPS generate numerous different isoprenoids produced by plants. These secondary modifications commonly include oxidation, reduction, isomerization and conjugation that change functional properties of terpenoid molecules.

All living organisms can be classified based on the metabolic pathway used to produce the precursors of isoprenoid biosynthesis. The mevalonate pathway (MVA) is common in archaea, some bacteria, fungi and animals. The non-mevalonate pathway, or 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP), was discovered in other bacteria and some algae. It is remarkable that plants use both MVA and MEP pathways that occur in the cytosol and plastids, respectively. Prenyl transferases and terpenoid synthases have been also found in both cytosol and plastids. In general mono-, di- and tetraterpenoids are preferentially formed in plastids from the precursors of the MEP pathway, while the majority of sesqui- and triterpenoids is synthesized in the cytosol using precursors from the MVA pathway. It is important to note that the division by biosynthetic origin is not complete, as there is exchange of IPP units between the pathways [22–24].

Since the formation of plant isoprenoids involves several sub-cellular, tissue and organ compartments [25], it requires intra- and possibly intercellular transport of

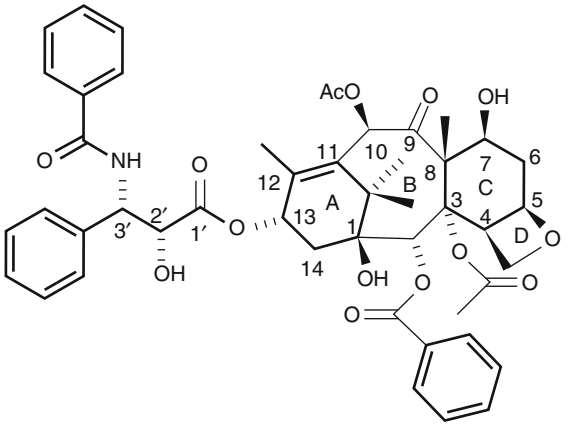




Ginsenoside	R^1	R^2	R^3
Rb_1	Glc(1–2)Glc	H	Glc(1–6)Glc
Rb_2	Glc(1–2)Glc	H	Ara(pyr)(1–6)Glc
Rc	Glc(1–2)Glc	H	Ara(fur)(1–6)Glc
Rd	Glc(1–2)Glc	H	Glc
Re	H	Rha(1–2)Glc -O-	Glc
Rf	H	Glc(1–2)Glc -O-	H
Rg_1	H	Glc -O-	Glc

Fig. 23.1 (**a**–**c**) Chemical structures of triterpenoids discussed in this chapter (Modified from Vasil'eva and Paseshnichenko [9] (**a**, **b**) and Smolenskaya et al. [14] (**c**)). (**a**) Spirostanol steroidal glycosides and aglycon (diosgenin). (**b**) Furoostanol steroidal glycosides. (**c**) Dammarane-type triterpene glycosides – ginsenosides. *Ara(pyr)* arabinopyranose, *Ara(fur)* arabinofuranose, *Glc* glucopyranose, *Rha* rhamnopyranose

a



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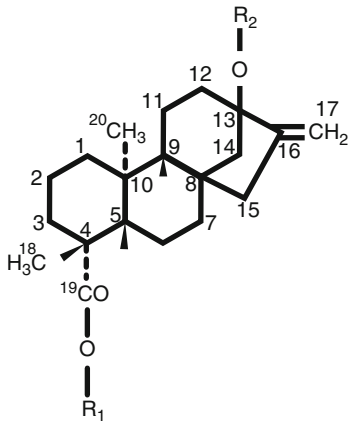

Compound	R_1	R_2
Steviol	H	H
Steviolbioside	H	Glc^{2}-^{1}Glc
Stevioside	Glc	Glc^{2}-^{1}Glc
Rebaudioside A	Glc	Glc^{2}-^{1}Glc |^{3} ^{1}Glc
Rebaudioside B	H	Glc^{2}-^{1}Glc |^{3} ^{1}Glc
Rebaudioside C	Glc	Glc^{2}-^{1} Rha |^{3} ^{1}Glc

Fig. 23.2 (**a**–**b**) Chemical structures of diterpenoids discussed in this chapter. (**a**) Paclitaxel. (**b**) Major steviol glycosides (From Bondarev et al. [15]). *Ac* acetate, *Glc* glucopyranose, *Rha* rhamnopyranose

intermediates, e.g. P450 enzymes involved in the modification of mono-, sesqui-, di- and triterpenoids are associated with the endoplasmic reticulum whereas final products are stored usually in vacuole or periplasmatic space of cells.

23.1.3 In Vitro Culture of Undifferentiated Plant Cells as a Biological System with Unique Secondary Metabolism

In vitro culture of somatic plant cells is a unique artificially created biological system representing a heterogeneous population of dedifferentiated cells. This system undergoes a continuous process of auto-selection, which depends on the intensity and stability of cell proliferation [26]. Both the physiological and genetic studies demonstrated a prominent difference between the cell in such an artificial population and in plant [7, 26, 27]:

- Cells in culture are truly dedifferentiated while in plant they perform specific and predetermined functions;
- Cells in culture are free from the organism control. Without the precise "directives" from plant signaling system cell development is switched to autoselection based on the intensive and stable proliferation;
- Cells in culture are heterogenic morphologically, physiologically, biochemically and genetically heterogenic. This heterogeneity enables flexibility required for the adaptation of proliferating population to *in vitro* conditions.

Figure 23.3 shows microphotographs of cells in suspension cultures obtained from various medicinal plants at the Department of cell biology and biotechnology, Timiryazev Institute of Plant Physiology (Moscow, Russia) as further discussed further in this chapter.

As a result of these unique cell characteristics secondary metabolism in the cell culture undergoes significant changes when compared to intact plant (Table 23.1).

In vivo biosynthesis of secondary metabolites is regulated by the plant signalling system and is not crucial for the survival of the individual cells [28]. In contrast, the auto-selection process in the dedifferentiated cell culture results in preferable production of metabolites that promote intensive and stable proliferation. Thus, biosynthetic pathways leading to the formation of secondary metabolites in cell culture is suppressed or arrested in the due course of the repetitive subcultures. Consequently, cell cultures that demonstrate active biosynthesis and accumulation of secondary products may be highly exclusive. However, there are a few basic principles that may enable intensive production of secondary metabolites in cultures *in vitro* cultures:

Principle 1. Cell cultures produce secondary metabolites that promote cell proliferation. Hundreds of different isoprenoid molecules are involved in plant stress response and adaptation mechanisms and some of them may benefit cell proliferation ability [3]. Between them one can found isoprenoids with remarkable antioxidative, osmoprotective, growth-stimulating and other activities inherited from their stress-defense function in the intact plant.

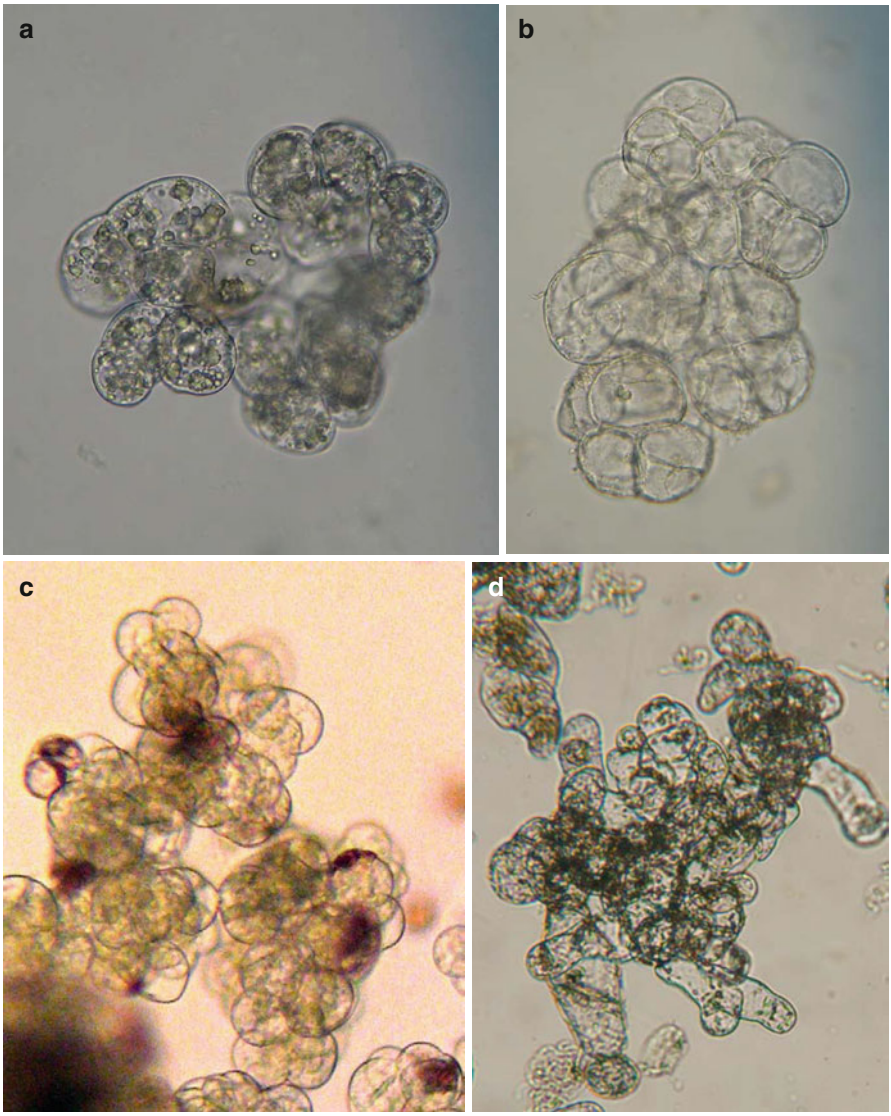

Fig. 23.3 (**a**–**d**) Photographs of dedifferentiated cells of *Dioscorea deltoidea* (**a**), *Panax ginseng* (**b**), *Polyscias filicifolia* (**c**) and *Taxus baccata* (**d**), grown as suspension cultures at the Department of Cell Biology and Biotechnology, Timiryazev Institute of Plant Physiology (Moscow, Russia) and discussed in this chapter

Principle 2. Recently it has been hypothesized that some secondary metabolites perform additional functions beside their main ecological role [29]. For example, on one hand, alkaloids protect plants from herbivore; on the other hand, they may be involved in nitrogen accumulation and storage similar to steroidal glycosides in dioscorea, which serve as sugar reserves. These additional functions may favour accumulation of such metabolites in the cell culture.

Table 23.1 Major differences in the production of secondary metabolites from cultured plant cells when compared to intact plant

Factors affecting secondary metabolite production	*In vitro* culture of undifferentiated cells	Intact plant
Cell differentiation and proliferation	Secondary metabolites are synthesized in continuously proliferating un-differentiated cells	Secondary metabolites are synthesized in differentiated non-proliferating cells
Cell ultrastructure	Scarcity of vacuoles and plastids in the population of meristem-like and/or parenchyma-like heterotrophic cells	Intense intracellular compartmentalization, so numerous organelles available for plastid-associated biosynthesis and storage of secondary metabolites
Cell compartmentalization and tissue-specificity	Both biosynthesis and accumulation of secondary metabolites are limited to either a single cell or 10–30 cell aggregates, or sequestrated to the culture medium	Biosynthesis of secondary metabolites is tissue-specific. Secondary products can be transported and stored in different organs

Principle 3. Production of secondary metabolites in plant cell cultures can be enhanced by the following physiological and biotechnological methods [7, 30]:

(a) Elicitation and/or short-term stress are the most common used techniques. The latter can be only effective if the target metabolite is a part of the inducible defence system. Other approaches include manipulating the plant growth regulators in culture medium, balancing medium nutrient composition and optimization of physical environment such as gas composition, light quality, etc.
(b) Due to high heterogeneity of cell population classical selection aimed at cells with abnormally elevated production of target secondary metabolites have been proved successful.
(c) A dramatic shift in cell metabolism caused by mutagenesis, reaction chain inhibitors, etc. followed by selection of cells with elevated metabolite production.
(d) Metabolic engineering, i.e. overexpression or silencing of genes encoding key enzymes of the specific metabolic pathways.

The vast majority of biotechnological studies utilising plant cell cultures for the production of bioactive organic compounds are focused on the effective methodology of increasing the content of target metabolites in dry cell biomass [7, 31]. This research can be significantly intensified by systematic and detailed investigation of factors affecting the formation of certain groups of secondary metabolites in plant cell cultures *in vitro.*

Below we discuss a systematic analysis of factors that influence the production of two main isoprenoid groups, di- and triterpenoids synthesised through different

pathways (MVA and MEP-associated) in cultures of dedifferentiated cells obtained from various medicinal plant species. Some of the data summarised here have been obtained in the course of 50 years of intensive research in authors' laboratories and is presented in English for the first time.

23.2 Steroid Glycosides in Plant Cell Cultures

23.2.1 Overview of Steroid Glycosides

Steroidal glycosides (SG) is a large group of organic compounds with C27 aglycones composed of cyclopentanoperhydrophenanthrene structure (rings A, D, C and D) and a modified side chain at C-17 position. Steroidal glycosides are classified into three groups based on the structure of their aglycones: (1) Spirostane-type, with a hexacyclic ABCDEF-ring system, (2) Furostane-type having pentacyclic ABCDE-ring system with the sixth open F ring and the least frequent, (3) Pregnane-type, a tetracyclic ABCD-ring system [32]. In spirostanol and pregnane-type glycosides a single carbohydrate chain is attached to a C-3 atom while furostanol glycosides carry two carbohydrate fragments at C-3 and C-26 positions. The additional glucose at C-26 position in furostanol glycosides dramatically changes their biochemical properties and physiological activities compared to other SG types. Furostanol glycosides are hydrophilic substances with pronounced immunostimulating activity and were proved effective in the treatments of patients with sex disorders. Spirostanol glycosides are hydrophobic molecules with well-known antimicrobial, anti-fungal, cytotoxic and anti-tumor activity [33, 34].

In plant cells, steroidal glycosides are synthesised from cholesterol in a series of oxidation reactions yielding furostanol structure, followed by glycosylation of hydroxyl groups at C-26 and C-3 carbon atoms [35, 36]. Interestingly, 25-S and 25-R epimers are formed at early stages of a biosynthetic pathway with no evidence for their subsequent epimerization. All steps of SG biosynthesis are performed in the cytosol in the association with endoplasmic reticulum. Furostanol-type SG are synthesised in leaves, some in acylated forms. The newly formed furostanol glycosides are transported through phloem to different organs, especially to storage, like tubers, to be further converted into spirostanol glycosides in the one-step reaction catalysed by the furostanol glycoside specific 26-O-β-glucosidase (F26G) [37]. Spirostanol glycosides can be accumulated in tubers at high quantities, up to 8 % dry weigh. Several studies suggested that furostanol glycosides could be also transported from leaf mesophyll to stem and leaf epidermis and stored in idioblasts that makes them unavailable for mesophyll-localized specific β-glycosidase [38, 39].

Spirostanol glycosides in underground organs and furostanol glycosides in the upper parts of plant have been shown to carry out protective function in constitutive and semi-inducible plant defence systems, respectively. Furostanol-type glycosides can be considered as non-toxic transport and storage form of SG within the semi-inducible plant defence system. Under pathogen attack the disruption of cell

membranes occurs, so, most furostanol glycosides are converted into spirostanol glycosides caused by mixing with F26G [39]. Spirostanol glycosides are the active and extremely toxic forms of SG and play an important role in constitutive defence mechanism as a "chemical weapon" against the invasive pathogens in storage organs. Non-toxic furostanol glycosides have lots of "positive" properties, such as antioxidant and membrane stabilisation and may show important side-functions in cell metabolism.

23.2.2 Steroid Glycosides (SG) in Cultures of Undifferentiated Plant Cells

First studies of SG in dedifferentiated cultures *in vitro* were presumably focused on their highly valuable aglycones. For decades diosgenin – an aglycone of many SG – was an irreplaceable substrate for pharmacological synthesis of steroid hormones such as cortisone, pregnenolone and progesterone [40]. Tubers of tropical vine, *Dioscorea deltoidea* Wall. were found to be the best natural source of this compound because, they accumulate diosgenin-based glycosides only. Soon after extensive harvesting of *Dioscorea deltoidea* plants in their natural habitats brought the species nearly to extinction and thus promoted the research on cell cultures as an alternative source of diosgenin for pharmacological industry [41]. However in the late 1990s, the interest declined caused by a discovery of economically effective production of steroid hormones by the microbial strains utilizing β-sitosterol, a cheap and available waste product of timber industry. In the twenty-first century research interest to SG production in plant cell cultures was refocused on the production of steroidal glycosides *per se*. SG are commonly used by both Western and traditional medicine to treat hypotension ("Diosponin", "Polysponin"), for strengthening ("Tribestan"), as immunomodulators and adaptogens as well as sex-stimulating drugs. Therefore standardization of SG-containing substrates for pharmacological industry is urgently required and is particularly important because plant material normally contains glycosides of both furostanol- and spirostanol-type that may produce alternative, if not the opposite, physiological effects in humans.

In 1970s–1980s SG and their aglycones have been discovered in cell cultures obtained from various plants including *Dioscorea* spp.: *D. deltoidea, D. tokoro, D. nipponica, D. composita* and *D. spiculiflora* [42–45], other genera like *Solanum* spp. [46, 47]; *Yucca* spp., *Agava* spp. [43], *Licopersicon* spp. [43] and specis *Trigonella foenum-graecum* [48], *Momordica charantia* [49] and *Costus speciosus* [50]. In the majority of studies SG content was estimated by content of their aglycones.

Cell cultures differ from intact plants by both quality and quantity of the produced SG. For example, gitogenin and manogenin were the main SG aglycones found in *Yucca glauca* cell culture while sarsapogenin, neotigogenin, gitogenin, marcogenin, tigogenin and smilagenin in intact plant [51]. Also fractions of various glycosides shifted in cell cultures compared to intact plant. For example, solasodine,

a steroidal pseudoalkaloid, was the major aglycone in *Solanum laciniatum* plants with diosgenin being a minor component. By contrast, cell culture of *S. laciniatum* produced diosgenin as a major compound, while solasodine content was reduced nearly to zero [52].

The effect of medium composition including various combinations of growth regulators, carbohydrate sources, the nitrate and ammonium ratios, phosphate content, etc., on SG content has been thoroughly investigated in plant cell cultures [53–57]. Some authors also suggested that cell differentiation status and morphogenesis changed SG production by plant cell cultures [58]. However despite the remarkable research interest no common pattern of SG formation in cell cultures in response to growth conditions has been found. We assume, based on the literature available for the last 40 years and our experience that regulation of SG biosynthesis in undifferentiated cell cultures is likely to be species- and compound-specific. Below, we describe a systematic approach to step-by-step improvement of diosgenin production in *Dioscorea deltoidea* cell culture. It was based on optimization of culture conditions and enabled the up-scaling of the process from the laboratory to semi-industrial bioreactors.

23.2.3 Steroid Glycosides in Dioscorea deltoidea *Cell Cultures*

The first cell cultures of *D. deltoidea* were obtained independently by Staba (USA) and Butenko (the former USSR) in late 1960s and was followed by detailed analysis of cell growth characteristics and optimization of culture media [27, 57, 59]. Interestingly, in both callus and suspension cultures, sucrose uptake from medium resulted in noticeable starch accumulation in protoplasts [60]. Cultivation of *D. deltoidea* suspension cell cultures in flasks and bioreactors was successful [61, 62]. Kaul and Staba were the first to demonstrate the ability of *D. deltoidea* cell cultures to produce diosgenin [59]. According to their records, diosgenin was synthesized intensively by undifferentiated cells while only trace amounts of the compound have been detected in rhizogenous, i.e. differentiated, tissues [59, 63]. Soon after, the first *in vitro*-produced steroidal glycoside (furostanol-type compound without its carbohydrate chain) was purified from hydrolyzed cell biomass of *D. deltoidea*.

Among pre-screened *Dioscorea* species cell cultures derived from *D. deltoidea* demonstrated the highest diosgenin production [44]. Diosgenin content varied from 3 to 30 $mg \cdot g^{-1}$ dry weight equivalent to 10–100 $mg \cdot g^{-1}$ dry weight SG content, respectively [54, 60]. Some data showed that diosgenin accumulation in cell culture occurred at the later phases of growth cycle [60, 61, 64]. On the contrary, Drapeau [42] reported diosgenin content to remain constant during the cultivation cycle.

Intensive research has been focused on the effect of the environment on diosgenin content in *D. deltoidea* cell culture. The ratio and concentration of ammonia and nitrate in culture medium were proved to be important [61]. Dioscorea cells were able to utilize various carbohydrate sources including sucrose, glucose, galactose, lactose and starch. However, the highest diosgenin content was observed at

4–5 % sucrose [65]. Manipulation of growth regulators in culture medium significantly altered the diosgenin production. It was maximized in presence of 2,4-D alone or in combination with IAA [54]. Supplement of diosgenin precursors such as mevalonic acid and cholesterol into the culture medium also improved diosgenin production [54].

Tal et al. [62] applied a biphasic cultivation to stimulate diosgenin biosynthesis in *Dioscorea deltoidea* cell culture. Increasing phosphate and sucrose concentration in culture medium at the exponential growth phase resulted in eight times increase in diosgenin content by the end of cultivation cycle from 0.4 % initial content. However, the overall productivity of the cell culture remained below 15 $mg \cdot L^{-1}$ medium [66].

Another approach leading to high diosgenin production in *Dioscorea* cell cultures was based on chemical mutagenesis followed by classical selection of cells by their proliferation intensity [67]. Ionizing radiation as mutagenic factor was found less effective. Below, we review the main growth characteristics of the selected *D. deltoidea* cell strains with elevated diosgenin and steroidal glycosides production.

23.2.4 Growth and Biosynthetic Abilities of the Selected Strains of Dioscorea deltoidea *Plant Cell Culture*

The initial cell line D-1 was obtained from *Dioscorea deltoidea* tuber in 1968. New strains were obtained as a result of exposure of D-1 cells to different concentrations of NMM (*N*-nitroso-*N*-methylurea) followed by cell selection by growth intensity [67]. The most promising strains resulted from 0.5; 1.0 and 8.0 $mM \cdot h^{-1}$ NMM treatments were named as DM-0.5, DM-1 and DM-8 respectively. Interestingly, line DM-8 was prototrophic and showed intensive proliferation on the medium without growth regulators.

Cytogenetic and Growth Parameters of the Selected *D. deltoidea* Cell Strains

After 5–7 days of culture chromosome number varied from 8 to 68 for D-1 strain, from 10 to 63 for strain DM-0.5, from 10 to 84 for strain DM-1 and from 9 to 80 for strain DM-8 compared to 20 chromosomes (at 2N = 2C) in cells of intact plant. Thirty to forty percent cells in strains D-1 and DM-0.5 were diploid and triploid while 45–50 % cells in strain DM-8 were diploid. The ploidy of the strains appeared stable under standard conditions, but was altered by changes in composition of culture medium. For example, after eight cycles of subculture in vitamin-free medium 30–40 % cells of strain DM-0.5 became polyploid while 40–45 % cells of strain DM-8 – haploid. When both cultures were transferred back to vitamin-containing medium their ploidy levels retained to the initial values [68]. These data

illustrate the flexibility of undifferentiated cell cultures and their high adaptability based on heterogeneity of the cells in population as well as the stability in constant cultivation conditions.

In all strains, except DM-8, 70–80 % cells formed aggregates of less than 20 cells. In prototrophic strain DM-8 60 % cells formed aggregates of over 20 cells [69].

Proliferation activity was different between strains. For example, in DM-0.5 strain fraction of proliferating cells (assessed by ^{3}H-thymidine accumulation) reached 80 % on the forth day of subculture, while in strain D-1 it remained below 65 % by the end of subculture cycle [70]. Both strains demonstrated two peaks of mitotic activity on the second to third and sixth to seventh day, but the mitotic index (MI) of DM-0.5 was 25–30 % higher than that of D-1 strain [27].

Remarkably, strain DM-0.5 retained the intensive proliferation ability for over 30 years of repeated subculture.

Further analysis revealed significant differences in the duration of mitotic cycles between the initial strain D-1 (26–28 h) and the mutant strain DM-0.5 (24–25 h). Mitotic cycle of DM-0.5 strain was shorter than D-1 due to shortened S and G2 phases (Fig. 23.5). However, the duration of actual mitosis was shorter in D-1 strain [70].

Under batch-cultivation in flasks and bioreactors all strains exhibited classical S-shape time-response curve of dry and fresh weight accumulation and cell count. All strains showed comparable duration of growth phases including the lag-phase from 0 to 6–7 days depending on the inoculum size, 4–6 days in the exponential phase and 1–3 days in the slow growth phase. The total absence of the stationary growth phase in all strains could be a special feature of *Dioscorea* cell culture. Increase in sucrose concentration in culture medium resulted in prolonged exponential growth up to 10–12 days.

The main growth characteristics of individual strains of *Dioscorea deltoidea* plant cell culture are summarised in Table 23.2 (according to [27, 71, 72]).

Steroidal Glycoside Content in Different Strains of *Dioscorea deltoidea* Cell Culture

Analysis of steroidal glycosides in cultivated cells of *D. deltoidea* demonstrated that in all strains overwhelming majority was represented by furostanol forms. Table 23.3 shows how production of SG varied between strains. Note that spirostanol-type glycosides content was less than 3 % of total SG.

To explain near absence of spirostanol-type glycosides in *D. deltoidea* cell lines, the additional experiments were performed such as water extraction enabling autofermentation of each strain were performed. During the procedure, the lyophilized cell biomass was extracted with water at 26 °C for 3 h, thus allowing endogenous β-glycosidases to remain active and convert furostanol glycosides to spirostanol forms. As a control, an extraction with 70 % methanol was performed, which resulted in the inactivation of all types of β-glycosidases. Table 23.4 shows how the content of furostanol glycosides was affected by the selective extraction.

Table 23.2 Growth characteristics of the selected *Dioscorea deltoidea* strains grown in flasks

Strain	Maximum dry mass accumulation, M (g L^{-1})	Growth index, I	Specific growth rate, μ (day^{-1})	Economic coefficient on sucrose, Y	Productivity on biomass, P (g L^{-1} day)	Doubling time, τ (days)
D-1	11.2	8.4	0.18	0.30	0.55	3.9
DM-0.5	11.9	8.4	0.20	0.33	0.66	3.5
DM-1	11.7	7.2	0.17	0.32	0.63	4.1
DM-8	10.9	6.8	0.16	0.35	0.60	4.3

Average of 15 replicates. Coefficient of variation (CV) $\leq$ 25 %

Table 23.3 Content of furostanol-type and spirostanol-type glycosides in *Dioscorea deltoidea* strains [73]

Strain	Glycoside content (% dry weight)		Spirostanol glycosides (% of total)
	Furostanol-type	Spirostanol-type	
D-1	9.3	0.07	0.8
DM-0.5	3.2	0.06	1.9
DM-1	2.0	0.04	2.0
DM-8	2.1	0.06	2.9

Table 23.4 Effect of extraction method on content of furostanol-type glycosides in strains of *Dioscorea deltoidea* cell culture [73]

Strain	Olygofurostanoside content (% dry weight)		Extraction loss (%)
	Extraction with 70 % methanol	Extraction with water (autofermentation)	
DM-0.5	7.8	7.8	0
DM-1	3.5	1.2	70
D-1	0.92	0.15	85
DM-8	1.7	0.24	85

According to the data reported in Table 23.4 the low content of spirostanol glycosides in DM-0.5 strain could be a result of low or inactive β-glycosidase in this strain. In contrast, water extraction of DM-1, D-1 and DM-8 strains resulted in 70–85 % loss of furostanol glycosides caused presumably by high β-glycosidase activity. Thus, total absence of spirostanol glycosides in those strains could be due to intracellular isolation of the enzyme and furostanol glycosides in different compartments.

Analysis of individual SG in all cell strains using RP-HPLC-UV, GS-MS after acid hydrolysis and IR spectroscopy showed that protodioscin and deltoside (aglycone diosgenin, 25R-configuration) and their 25-S-isomers (aglycone yamogenin, 25-S-configuration) were detected as major compounds. Interestingly, 25-S-isomers were not found in *D. deltoidea* intact plants, but have been detected in other *Dioscorea* species [9].

Quantitative analysis of SG content by UV–VIS spectrophotometry, HPLC and GC after acid hydrolysis showed similar results with less than 20 % difference

Table 23.5 Content of furostanol-type glycosides in strains of *Dioscorea deltoidea* cell cultures, $mg \cdot g^{-1}$ dry weight [73]

Strain	Total content	25R-configuration		25S-configuration		25S to 25R ratio (%)
		Protodioscin	Deltoside	S-Protodioscin	S-Deltoside	
D-1	14.2	4.2	1.8	5.5	2.7	58
DM-0.5	92.3	54.3	27.1	7.2	3.7	12
DM-1	57.6	31.2	20.9	3.4	2.1	9
DM-8	29.2	9.1	10.2	5.0	4.9	34

Average of 15 replicates. CV ≤ 35 %

between the methods. Table 23.5 shows SG content in the selected strains at the early-stationary phase of growth (14–18 days of culture).

Highest furostanol glycoside content, about 12 % dry weight, was recorded in DM-0.5 strain followed by 7, 4 and 2 % in DM-1, DM-8 and D-1, respectively. Ratio of 25-R and 25-S isoforms remained constant and specific for each strain. The content of SG in DM-0.5 strain was higher than in tubers of *D. deltoidea* plants.

It is important that furostanol glycoside content remained constant at the initial stages of culture cycle, but increased 1.5–2 folds in transition from the exponential to the stationary phase suggesting the continuous synthesis of SG in cell cultures *in vitro* cell cultures [74].

In each strain SG content and accumulation pattern remained stable for 40 years of maintenance by periodic subcultures. Figure 23.4 shows HPLC profiles of different strains of *D. deltoidea* cell suspension culture maintained in flask in the year 1991 and DM-0.5 strain cultivated in 630 L bioreactor after 20-year interval.

Intracellular Localization of Steroid Ginsenosides in *Dioscorea deltoidea* Cell Cultures

Information on intracellular localization of SG can help to increase the productivity of cell cultures. In the first series of experiments SG content was compared between cells and protoplasts isolated from the same culture to reveal possible SG localization in the cell wall and/or in the periplasmic space. The results showed that over 50 % of total SG accumulated in periplasmic space. In 7-days-old culture, only 7.5 $mg \cdot g^{-1}$ glycosides of total 35 $mg \cdot g^{-1}$ SG were found in protoplasts. With the ageing of culture SG content in protoplasts increased to 20 $mg \cdot g^{-1}$ compared to 50 $mg \cdot g^{-1}$ in periplasmic space, presumably caused by their accumulation in vacuoles [73].

SGs are electron-dense substances and electronic microscopy indicated their intracellular localization. Fixation with glutaraldehyde followed by osmium tetroxide staining was used to prevent SG elution from cells. Electronic microphotographs prepared by this method showed localization of electron-dense substances, which were likely to be SG, in vacuoles, cell walls and inter-cellular spaces. In contrast, other fixations resulted in SG elution from cells and absence of electron-dense compounds in the microphotographs. It is important that distribution of electron-dense

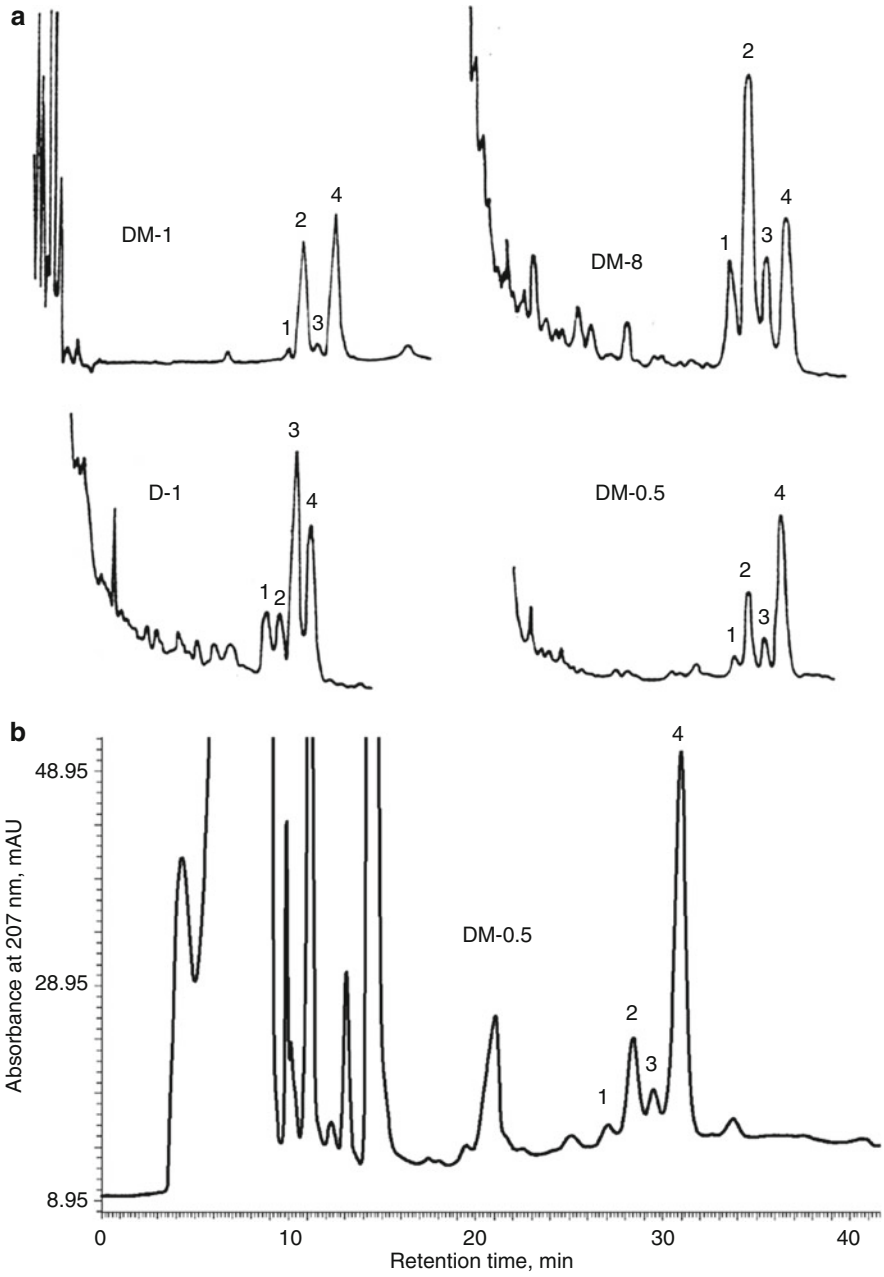

Fig. 23.4 (**a**–**b**) RP-HPLC-UV chromatograms of saponins from *Dioscorea deltoidea* cell suspension cultures. (**a**) Different strains of *D. deltoidea* cell suspension culture, chromatographic analysis made in 1991 [73]. (**b**) *D. deltoidea* cell suspension culture strain DM-0.5, chromatographic analysis made in 2007. *1* 25(*S*)-deltoside, *2* 25(*R*)-deltoside, *3* 25(*S*)-protodioscin, *4* 25(*R*)-protodioscin

compounds between cell compartments was specific for every cell strain. For example, in modest glycoside producers D-1 and DM-8, SGs were detected mainly in vacuoles and cell walls, respectively [75].

Optimization of Culture Medium for Steroidal Glycoside Production in *Dioscorea deltoidea* Cell Cultures

Optimization of culture medium on biomass and SG production in *Dioscorea deltoidea* cell suspension was based on variation in IAA, NAA and 2,4-D concentration, source of carbohydrate and composition of mineral elements. Without growth regulators cell growth was totally arrested at the beginning of the second subculture. Supplementation of auxins into culture medium was essential for the cell growth. Among all auxins studied, addition of 2,4-D to the culture medium was most effective and resulted in 1.5–2.0-fold increase in SG production. The exact mechanism of phytohormone effect on SG biosynthesis in cell suspension remains unknown. The pronounced beneficial effect of 2,4-D may be due to its high ability to intensify the proliferation and dedifferentiation of cultured cells [74, 76, 77].

Biomass accumulation in cell culture increased with the increase in sucrose concentration in the medium from 3 to 5 %, while specific growth rate and sucrose uptake remained unaffected. However at the end of subculture cycle decrease in SG production was recorded for cell suspension in sucrose-enriched medium. Interestingly, in sucrose-enriched medium the portion of 25-S glycoside to total SG increased by 50 %, as well as deltoside to protodioscin ratio. Thus, the increase in sucrose concentration from 3 to 6 % caused a remarkable shift in S-shaped growth curve due to the prolonged exponential phase, elevated accumulation of dry biomass and reduction in SG content; at the same time culture productivity calculated as total SG production remained unchanged [78].

Threefold increase in phosphate in culture medium did not bring any significant changes in productivity of DM-0.5 cell line, and phosphate uptake of 0.4–0.5 mM · day^{-1} was similar to cell culture in standard medium. In contrast to phosphate, nitrogen source affected both cell growth and production of steroidal glycosides. At the standard NH_4^+ to NO_3^- ratio (20:40 mM) nitrogen uptake did not exceed 50 % of total nitrogen content. However, the decrease of both ammonium and nitrate by 50 % resulted in total arrest of culture growth. Decrease only in NO_3^- concentration by 50 % (NH_4^+/NO_3^- = 1:1, 20:20 mM) enabled stable cell growth but resulted in decline of SG production by 4–5 %. At the end the ratio of NH_4^+/NO_3^- was optimized as 1:3 in equivalent of 40 mM total nitrogen [78]. Therefore, optimization of nitrogen source and concentration had the most prominent impact on SG production in *D. deltoidea* cell culture.

Effect of Cultivation Mode on Steroidal Glycoside Production

To investigate the effect of cultivation mode (callus or suspension culture) on SG production 10–12 callus cell lines were obtained from all *D. deltoidea* strains [79]. Table 23.6 summarises the results of SG biochemical analysis in all callus lines of different strains.

Table 23.6 Content of furostanol glycosides in *Dioscorea deltoidea* strains cultured on agar medium ("on-top" cultivation), mg g^{-1} dry weight (summary for all cell lines)

Strain	Total content	25R-configuration			25S-configuration		
		Proto-dioscin	Delto-side	Protodioscin to deltoside ratio	S-proto-dioscin	S-deltoside	Percent of S-configuration
D-1	5.5	1.1	1.4	0.8	1.7	1.3	54
DM-0.5	21.8	13.1	8.7	1.5	0	0	0
DM-1	28.4	13.5	7.5	1.8	4.8	2.6	26
DM-8	6.8	2.3	2.8	0.8	1.1	0.6	25

Mean values, CV ≤ 40 %

Furostanol glycosides varied within the following limits: 0.4–1.0 % DW for D-1 strain, 0.6–3.3 % DW for DM-0.5, 1.2–4.2 % DW for DM-1 and 0.2–1.4 % DW for DM-8. Therefore, total SG production in *D. deltoidea* callus cultured on agar medium was three to ten times lower than that in suspensions in liquid medium. The most significant loss in SG content was recorded for DM-0.5 strain, which has been proved the most productive in suspension cultures. Pattern of biosynthetic productivity of the cell lines cultured on solid medium resembled those of suspension cultures: DM-1 and DM-0.5 strains produced three to four times higher amount of SG than D-1 and DM-8 strains. However, DM-1 cell culture on solid medium was more productive based on SG content of DM-0.5; opposite relations were observed for these lines in suspension.

Protodioscin to deltoside ratio was slightly shifted in callus culture when compared to suspensions. For instance, deltoside became a major glycoside in D-1 strain. The 25-S-glycosides fraction was not detected in DM-0.5 strain but its content increased up to 25 % in DM-1 strain.

Therefore, culture mode affected the SG content in all strains resulting in significant decrease in SG in callus culture when compared to suspension. Also biomass productivity appeared to be two to three times lower on solid medium than in liquid medium, possibly caused by shorter subculture cycle.

Hence we describe below the experiments which show changes in the cell growth related to SG production.

Effect of Cultivation Regime on Growth and Biosynthetic Characteristics of *Dioscorea deltoidea* Suspension Cell Cultures

According to the available literature both growth and biosynthetic traits of cell cultures could be strongly affected by bioreactor mode of operation such as batch, fed-batch and continuous (chemostat, turbidostate and auxostat). This effect is yet to be understood. In our study both D-1 and DM-0.5 strains retained their main growth characteristics when cultured in MF-107 bioreactor (New Brunswick, USA) operated as chemostat. However, maximal dilution rate (D), which equalled maximal specific growth rate (μ) of a strain, was higher for DM-0.5 compared to D-1: at $D = 0.22$ day^{-1} cells of D-1 were gradually eluted from the bioreactor, while DM-0.5 strain retained its growth characteristics [80].

The productivity of DM-0.5 and D-1 strains under the chemostat conditions was 2.3 and 1.8 $g \cdot L^{-1}$ medium per day, respectively, that is two to three times higher than their productivity under the batch conditions. Both strains fully retained their ability to biosynthesise SG under the chemostat conditions. The highest content of furostanol glycosides in DM-0.5 strain was 6 % DW which was lower than under the batch conditions. On the contrary, SG production in D-1 strain cultured under chemostat regime with a low dilution rate was higher than that under the batch regime. The proportion of individual SG such as protodioscin, deltoside and their 25-S-isoforms under chemostat regime remained unchanged when compared to the batch cultivation. Thus, we can conclude that chemostat regime is preferable for DM-0.5 and D-1 strains of *D. deltoidea* when SG productivity is considered: after strain-specific alterations in chemostat regime individual SG content was 1.4–1.6-times higher than that under the batch conditions, possibly due to more intensive cell growth under chemostat conditions [80].

In contrast to microbial cells dedifferentiated plant cells cultured under chemostat regime showed a higher productivity than it was expected based on mathematical models and it was assumed that it may be due to a remarkable change in cell population structure described below. However, the long-term cultivation of plant cell culture in chemostat in a standard medium using high dilution rate is impossible. Dramatic decrease in cell viability has been recorded for both strains after 30–40 days in chemostat with dilution rate $D = 0.20–0.22$ day^{-1}, that was followed by the reduction of growth intensity and subsequent death of the entire population.

Similar to *D. deltoidea* strains the complete loss of viability was reported in tobacco cells after 2 weeks in chemostat. However, it was shown that it was possible to extend duration of cultivation in chemostat up to 70 days by increasing the concentrations of all components of the medium [81].

Our experiments revealed the key role of phosphate in this process. Cell suspension of D-1 strain was cultured in flasks under semi-continuous regime using MS-medium with standard (1.25 mM) and elevated (2.94 mM) phosphate concentration. When cultured in phosphate-enriched medium, the cell suspension showed stable growth for 200 days at specific growth rate (μ) above 0.3 day^{-1}. On the contrary, in the standard MS medium specific growth rate as high as $\mu = 0.23$ day^{-1}, could be maintained only for 40 days, which was equal to 50 cell generations. After this period within several days cell viability was completely lost. Transfer of cell culture to standard medium after 130 days in phosphate-enriched medium caused nearly immediate death of the entire population [82].

The main reason behind the observed instability of cell proliferation and viability under continuous cultivation regime was a dramatic change in the population structure. At a high dilution rate the percentage of intensively dividing cells in the population increased because of rapid elution of cells which stopped growing or which showed slower growth ($\mu < D$). This inevitably resulted in depletion of culture heterogeneity which underpins the population flexibility and stable growth. The rapidly growing and dividing cells that remained in the population were very sensitive and fragile and were unable to survive for long.

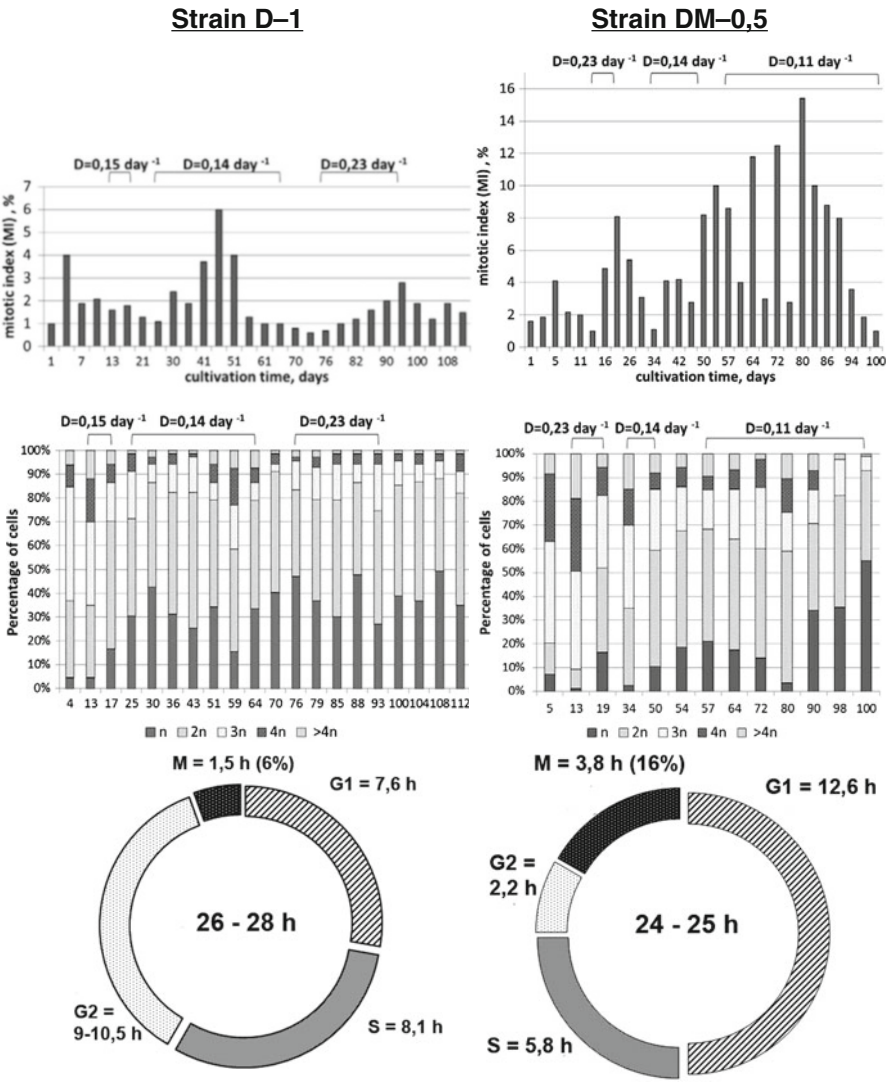

Fig. 23.5 Mitotic index, distribution of cells with different ploidy and duration of mitotic cycle phases in *D. deltoidea* suspension cell culture strains D-1 and DM-0.5 during long-term cultivation in "batch – continuous" regime. *Upper Square brackets* indicate chemostat regime with dilution rate D. n, 2n, 3n, 4n – ploidy of cells

This observation was supported by the results of further experiments with long-term cultivated (over 100 days) D-1 and DM-0.5 strains. These experiments were performed in bioreactors designed to switch between batch and chemostat mode at different dilution rates [83]. It was shown that a step-wise switch from the continuous to batch mode induced significant changes in cell culture (Fig. 23.5). In particular,

a specific growth rate under the batch regime increased due to accumulation of intensively proliferating cells during previous chemostat phase. This was associated with three to fivefold increase in mitotic index in chemostat culture compared to batch. The highest recorded mitotic index for D-1 and DM-0.5 strains under chemostat condition was 6 and 16 %, respectively. Interestingly, mitotic index correlated with the relative phase duration of mitotic cycle: in D-1 strain actual mitosis lasted for 6 % of the cell cycle, in DM-0.5 – 16 %. Assuming that in chemostat culture cell division was not synchronised, equality of mitotic index to the length of mitotic phase indicated that all cells in the population were constantly dividing. As a result, there were no cells in the population which "rested" in between the division circles. This change in cell " cell behaviour" explained the difference of cell cycle parameters in experiment compared to the mathematically modelled ones based on classical chemostat theory. Interestingly, sharp increases in mitotic index were followed by deep decreases down to 1–2 %, confirming our statement that the intensively growing cell population is very unstable. Possibly, cells in cell culture require a rest-period between divisions to produce stable and viable population.

The most dramatic change occurred in cell population under chemostat conditions was the change in ploidy level. In both strains, the proportion of haploid cells increased from 1 to 2 % at the beginning of chemostat conditions to 45–50 % at the end (~100 days). We suggested two possible reasons for that increase. First, it could be due to the lack of phosphate for DNA synthesis and second, that intensive division of cells with a minimal DNA content led to their accumulation in population. It is known that haploid cells are the most sensitive to culture conditions, thus their accumulation may result in significant decrease in culture viability.

Also changes in SG content in cell population under chemostat conditions were likely brought about by changes in cell population. After 30 days in chemostat at $D = 0.14$ days^{-1} furostanol glycosides content in D-1 strain decreased from 1.7 to 1.2 %, but then increased back to 2.7 % [83].

Thus, cell culture in continuous mode bioreactors is a powerful tool to study the unique properties of the cell *in vitro* including biosynthesis of secondary metabolites, because it allows regulation of cell physiology and population structure. For example, our results suggested that a "rest period" between mitotic cycles is necessary for stable cell proliferation and culture growth. Accumulation of intensively proliferating cells caused a deterioration of population heterogeneity, which made it vulnerable in chemostat conditions and led to entire loss of cell viability within a few subculture cycles.

Unfortunately, we have to conclude that chemostat regime despite its higher productivity compared to other culture regimes was not a suitable tool for industrial production of cell biomass.

In general, we found that quantitative and qualitative content of SG in *D. deltoidea* cell cultures under different culture conditions were strain-specific and differed from those in intact plants.

1. Predominant biosynthesis of furostanol-type SG in cell culture was not affected by the presence or changes in activity of endogenous β-glucosidase, but in a

mutant DM-0.5 strain, which showed the highest SG production, the activity of this enzyme was undetectable.
2. Cell cultures of *D. deltoidea* were able to produce glycosides with S-configuration of C-25 carbon atom in their aglycone (yamogenin). These compounds were not found in intact *D. deltoidea* plants, but they have been detected in other *Dioscorea* species
3. All tested cell lines showed similar HPLC profiles of SG, with protodioscin and deltoside found to be the major SG in *D. deltoidea* cell cultures. It is a perfect example for totipotency of plant cells, because in intact plants protodioscin accumulated is seen in leaves and deltoside in tubers, while in cell culture both these SG are co-accumulated.
4. In *in vitro* plant cells SG were accumulated in periplasmic space and vacuoles and the accumulation in vacuoles increased with the ageing of culture.
5. SG content in cell cultures remained stable. The most productive DM-0.5 strain retained the ability to produce SG at the level of 5–12 % during 40 years of observation without any "rejuvenating" treatments.
6. Optimization of culture medium by adjusting the concentrations of growth regulators and mineral nutrition had almost no effect on SG content in *D. deltoidea* cell culture. The maximal increase in SG production due to medium composition was 1.5-fold. On the contrary, change of auxin type and bioreactor culture regime improved SG production suggesting a strong physiological correlation between SG biosynthesis and cell proliferation.

In intact plants SG play a role in protection from stress, so their biosynthesis and function is regulated at the organismal level. Therefore one can expect that SG biosynthesis in cell cultures *in vitro* will be reduced or even stopped. Unexpectedly it remained stable for over 40 years in *D. deltoidea* cell lines. Based on this funding we hypothesize that furostanol-type SG may act alternatively as potential antioxidants and membrane stabilizing agents in dedifferentiated cell population when compared in intact plants, so their biosynthesis is retained to support the culture growth (see "***Principle 1***" in Sect. 23.1).

23.3 Triterpene Glycosides (TG) in Plant Cell Cultures

23.3.1 Overview of Triterpene Glycosides

Tritepenoids are a large group of isoprenoid compounds synthesized from 2,3-oxidosqualene, the common precursor of triterpenoids and steroids. Intact plants contain free triterpenoids, triterpenoid esters of different organic acids and glycosides. The latter can be acylated at different positions of the aglycone and/or carbohydrate chains [11]. Among all triterpenoids triterpene glycosides form the largest group. Apart from aglycone the carbohydrate chains contribute to their vast structural diversity. More than ten different classes of triterpenoids serve as

aglycones in TG. Those from the oleanane, ursane, lupane and dammarane classes are the most widespread in higher plants [10].

Similar to SG formation, biosynthesis of triterpenoids and their glycosides is accomplished *via* a few homotypic stages as following: oxidosqualene cyclization, introduction of a limited number of oxygene-based function groups and attachment of one or more carbohydrate moieties [84]. For instance, the biosynthesis of the ginsenoside Rb_1 includes six sequential steps catalyzed by different enzymes [85].

All enzymes of TG biosynthesis are localized near ER membrane, alongside with the enzymes catalyzing the biosynthetic pathways of their common precursor, squalene [11]. Several reports suggested that individual stages of TG biosynthesis are combined to form metabolic complexes, or metabolomes, which facilitate both spatial and temporal regulation of TG formation [86]. Moreover, as demonstrated for some TG the genes coding the enzymes of TG biosynthesis are organized in clusters within a single chromosome, so they could be expressed in a coordinated manner [87, 88].

Functions of triterpenoids in plants are yet to be elucidated though there is a wealth of evidence pointing out the participation of triterpenoids in ecological physiology of plants, in particular, in the defense system [11, 84].

23.3.2 TG in Cell Cultures of Different Plant Species

Investigation of TG in plant cell cultures started in the middle of the last century. A considerable amount of information about biosynthesis of these compounds in callus and suspension cultures of different plant species was obtained (Table 23.7). The majority of TG found in plant cells cultured *in vitro* belong to the most widespread oleanane and dammarane classes. Together with the glycosides free TG aglycones, including oleanolic, ursolic and betulinic acids and their derivatives, were found [89, 90].

Qualitative Composition

Qualitative composition of TG in cultured cells and intact plants differed significantly. Also novel compounds lacking in the intact plants could be biosynthesized in the cell culture. For example, the callus cultures of *Akebia quinata* and *A. trifoliata* (Lardizabalaceae) were shown to accumulate rare 30-noroleanane-type glycosides which are not found in the intact plants [92, 109]. Furthermore the ratios of different glycoside groups were often altered when compared to the source plants. For example, it was found that the *in vitro* cells of *Glycyrrhiza glabra* (Fabaceae) were incapable of accumulation of glycyrrhizin, the main TG of the licorice tubers [97]. At the same time, these cultures accumulated substantial amount of soyasaponins, which in the intact plant were biosynthesized as a minor component of TG

Table 23.7 Triterpene glycosides (TG) in plant cell cultures obtained from different plant species

Species	Cell culture type	TG found	References
Aesculus hippocastanum	Callus	Escin	[91]
Akebia quinata	Callus	Glycosides of 30-noroleanolic acid and 30-norhederagenin	[92]
A. trifoliata			
Bacopa monnieri	Callus and suspension	Bacosides	[93]
Bupleurum falcatum	Callus and suspension	Saikosaponins	[94]
Calendula officinalis	Suspension	Glucosides and glucuronides of oleanolic acid	[95]
Centella asiatica	Callus and suspension	Asiaticoside, madecassoside	[96]
Glycyrrhiza glabra	Callus and suspension	Soyasaponins I and II, glycyrrhizin	[97]
G. uralensis			[98]
Gymnema sylvestre	Suspension	Gymnemic acid	[99]
Gypsophila paniculata	Suspension	Gypsogenin-3-*O*-glucuronide	[100]
Medicago sativa	Callus and suspension	Glycosides of syasapogenol B and medicagenic acid	[101]
M. truncatula			
Panax ginseng	Callus and suspension	Ginsenosides	[102]
P. notoginseng			[103]
P. japonicus			[104]
Phytolacca americana	Callus	Phytolaccosides A, B, D	[105]
Polygala amarella	Callus	Polygalasaponin XXVIII and other presenegenin glycosides	[106]
Primula veris	Callus and suspension	Primula acid I	[107]
Stauntonia hexaphylla	Callus	Glycosides of 30-noroleanolic acid and 30-norhederagenin	[108]

mixtures only in the underground organs and at certain stages of the ontogenesis. Later studies employing highly sensitive methods such as competitive ISA and HPLC/MS showed that several licorice species were able to biosynthesize glycyrrhizin in callus [98] and suspension [110].

There are also examples of variation in qualitative composition of free triterpenoids in plants and their corresponding cell cultures. Cell culture of *Taraxacum officinale* (Asteraceae) synthesized α- and β-amyrin derivatives found only in the intact plants, but lacked taraxasterol – an essential component of intact plant lactifier [111]. A similar example was reported for *Euphorbia characias* (Euphorbiaceae) cell culture [112], as well as *Eucalyptus perriniana* (Myrtaceae). The latter biosynthesized novel and unique triterpenoids, e.g. C-23-hydroxylated oleananes and ursanes [113]. Changes in tissue specificity of certain reactions in the triterpenoid biosynthesis were also detected (e.g. cell cultures of certain Actinidiaceae species exclusively produced C-24-OH oleananes and ursanes whereas the intact plants

often contained mixtures of C-23/C-24-hydroxylated derivatives of these compounds [89, 90]).

Quantitative Composition

The TG content in cell cultures could vary significantly. Usually TG content is considerably lower in cells cultured *in vitro* compared to intact plants [98, 107, 110]. Often the ability to synthesize TG declined or even disappeared after prolonged cultivation *in vitro* cultivation [107], though exceptions exist. An example of such case could be observed with *Centella asiatica* (Apiaceae) suspension culture, which accumulated asiaticoside in higher amounts than in callus and intact plant [114, 115].

The maximum TG content in plant cell cultures was observed at the end of the exponential/beginning of the stationary phase of the cultivation cycle [98, 107, 110]. Similar pattern was reported for many free triterpenoids [111]. However, there are some exceptions, for example, marigold suspension culture featured two maxima of the accumulation of oleanane-type glycosides: in the beginning of exponential and in the middle-to-the-end of stationary growth phase [116].

Culture medium composition, namely specific phytohormones and their ratio, affected quantitative composition of TG in cell cultures [117]. The same was shown for the precursors of the TG biosynthesis [95], elicitors [99, 118] and stress hormones [117, 119]. Interestingly, the rate of biosynthesis of various triterpenoids by the same cell culture could vary significantly depending on additives to the cultivation medium. For instance, the addition of yeast extract to the cell-suspension culture of *G. glabra* led to the increase in betulinic acid formation and suppression of biosynthesis of soyasaponin, whereas methyl jasmonate had the opposite effect. It is possible these changes occurred due to the differences in regulation of biosynthesis of different triterpenoid groups [97].

Unfortunately, it was impossible so far to generalize on the pattern of TG formation in plant cell cultures *in vitro*. The reason is the scarcity and fragmentation of the knowledge. The only exception is the ginseng cell cultures, in which TG formation has been systematically studied for over 40 years.

23.3.3 Triterpene Glycosides in Ginseng Cell Cultures

The legendary ginseng is a representative of the relic genus *Panax* from Araliaceae family of higher plants [120]. Due to its unique therapeutic properties (adaptogenic, anti-inflammatory, immunomodulatory, neuroprotective, antitumor, etc.) ginseng became one of the most studied medicinal plants in the world [85].

Investigation of ginseng from the phytochemical standpoint lasted for more than 150 years. Different classes of secondary metabolites were isolated from

Panax spp.: polyacetylenes [85], sesquiterpenoids [121], unusual amino acids [122], alkaloids [123], etc., albeit the most typical of ginseng are the TGs of dammarane series – ginsenosides [124]. There is a large body of conclusive evidence supporting the crucial role of ginsenosides in majority of ginseng therapeutic effects [85, 124].

The ginsenosides are traditionally divided into two major classes [125]: glycosides of 20 (S)-protopanaxadiol (the Rb ginsenoside group: Rb_1, Rc, Rb_2 and Rd) and glycosides of 20 (S)-protopanaxatriol (the Rg ginsenoside group: Rg_1, Re and Rf).

The aglycones of these ginsenosides differ by a single hydroxyl group at the sixth carbon atom. The pronounced dissimilarity of the ginsenosides Rg- and Rb-groups in terms of their physicochemical and biological properties stemmed from this hydroxylation. In particular, C-6 hydroxylation with subsequent glycosylation at this position renders the ginsenosides of Rg-group, which are more polar and less toxic in comparison with the ginsenosides from Rb-group [126, 127]. These two groups of ginsenosides also differ significantly in exerting their biological activity, in most cases, the opposite effect. For example, most of the Rg-group ginsenosides show hypertensive and stimulatory effects on central nervous system. By contrast, glycosides from the Rb-group demonstrate sedative and hypotensive effects [128].

Till date, more than 300 different ginsenosides are isolated from different ginseng species [124]. Seven of those (Rg_1, Re, Rf, Rb_1, Rc, Rb_2 and Rd) were first obtained from the roots of *Panax ginseng* and are considered as major ginsenosides [129]. Among the oleanane-type glycosides the ginsenoside R_0 is the most widespread among different ginseng species [85, 125].

Physiological Characteristics of Cell Cultures of Different Ginseng Species

The first callus culture of *P. ginseng* cells was obtained in 1950s from the root of a 4-year-old plant from a plantation at South Sakhalin, USSR [130]. The first suspension cell culture from *P. ginseng* was derived from the callus of a cambial origin in 1970 [131].

After that, numerous callus and suspension culture lines have been obtained from various ginseng species, for example, *P. quinquefolius* [132], *P. japonicus* [104], *P. japonicus* var. *repens* [133], *P. notoginseng* [103], *P. vietnamensis* [134] and *P. sikkimensis* [135].

A vigorous and intensive growth was the basic characteristic of ginseng cell cultures regardless the origin. The optimum cultivation cycle for callus and suspension cultures was 26–30 and 14–21 days respectively, growth index varied from 5 to 12 and specific growth rate at the exponential growth phase varied from 0.12 to 0.23 $days^{-1}$ [136].

Study of ultrastructure of suspension cells showed that in the beginning of the stationary phase *P. ginseng* cells had the structural features of secretory cells, which

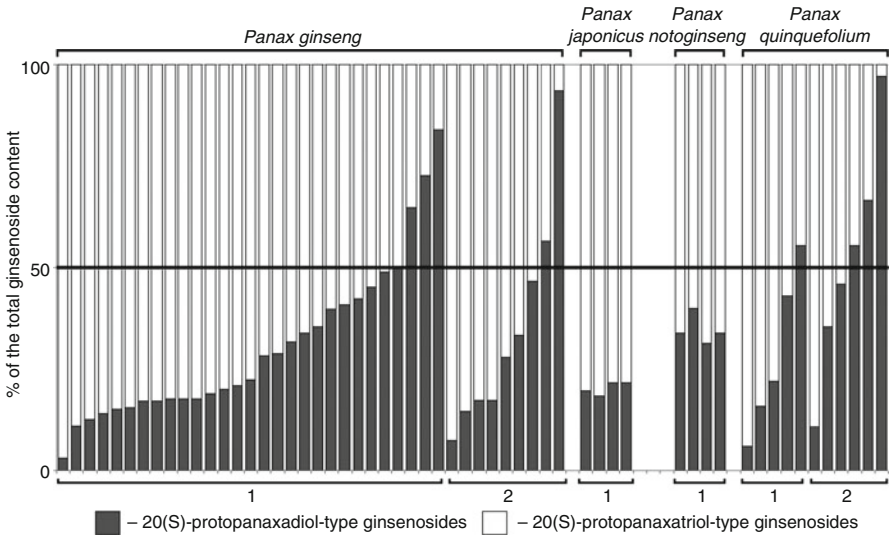

Fig. 23.6 Contribution of the major groups of dammarane-type glycosides in the total ginsenoside content in cell cultures of different ginseng species (according to the literature for the 1973–2013 period). *1* callus cell culture, *2* suspension cell culture

effuse lipophilic compounds to the vacuole. These features included (i) well-developed smooth ER, (ii) plastidial reticular sheath, (iii) osmiophylic depositions in nuclear and plastid envelopes, membranal structures and tonoplast [137]. The osmiophylic matter was deposited in vacuoles as a layer or globules and suggested to be of isoprenoid origin.

Ginseng cell cultures were found to be more sensitive to temperature than other plant species. For a number of *P. ginseng* cell strains increasing the temperature from its optimum by 2.5–4 °C resulted in 30 % decline in culture growth [138].

Ginsenoside Content in Different Ginseng Cell Cultures

The total ginsenoside content and its changes induced by culturing *in vitro* displayed the same relationship as are described above for the cell cultures of other species and TG groups [132, 139–142].

Regarding the ratio of the two major ginsenoside groups ginseng cell cultures *in vitro* were often characterized by predominance of 20 (S)-protopanaxatriol-type ginsenosides (Rg-group) (Fig. 23.6). Accumulation of the 20 (S)-protopanaxadiol-type ginsenosides (Rb-group) was sometimes unstable. Moreover there are indications of a considerable decrease of the ginsenoside diversity during long-term cultivation of *P. ginseng* mainly at the expense of Rb-group [143, 144].

Studies of ginseng cell cultures *in vitro* were focused on the effect of various cultivation conditions and stimuli on qualitative and quantitative composition of

ginsenosides. The chemical stimuli include: variation in cultivation media composition [103, 145, 146], addition of precursors and inhibitors of the isoprenoid biosynthesis pathway [147, 148] and plant stress hormones and different elicitors [149–151]. Among the physical stimuli the effects of aeration [152, 153], light [154] and ultrasound [155] on the ginsenoside composition were described. The majority of the stimuli studied, however, showed no or insignificant effect on culture growth and ginsenoside production. Substantial effects were reported for treatments with certain phytohormone combinations, jasmonates and elicitors [141, 146, 147].

It is difficult to generalize the patterns of TG biosynthesis and accumulation in plant cell culture *in vitro* based on published data, because obtained information comes from different cell lines and strains cultivated in various media and conditions. However, there are a few long-term systemic studies of a particular ginseng culture. Cell cultures of *P. ginseng* and *P. japonicus* var. *repens*, discussed below, serve as an example of such thorough study.

Comparison of Growth and Biosynthesis of Ginsenosides in *P. ginseng* and *P. japonicus* var. *repens* Cell Cultures

A comparative study of growth and ginsenoside production in the suspension cell cultures of *P. ginseng* C.A. Mey. and *P. japonicus* C.A. Mey. var. *repens* Maxim. obtained in 1998 was carried out in the authors' laboratories [133, 143]. Cell culture of *P. japonicus* var. *repens* was obtained from the radix of a 2-year intact plant harvested in Primorsky Krai, Russia; cell culture of *P. ginseng* was obtained from the lateral roots of a 6-year plant from a plantation belonging to 'Ginseng and Tobacco Company', South Korea. Importantly the standard cultivation media (MS with White vitamins) differed in hormone composition: *P. ginseng* was grown on 2,4-D and BAP, but *P. japonicus* var. *repens* on NAA and kinetin.

Growth and Physiology of the Cultures

The growth index and maximum mitotic index of the *P. japonicus* var. *repens* cell culture were found to be 1.5–1.8 folds higher than that of *P. ginseng* (6–7 and 3.5 %, respectively). The peak of mitotic activity was observed on the fifth to sixth day of cultivation in both the species. The number of viable cells was similar in both the cultures ranging between 87 and 90 %. Cell suspension of *P. japonicus* var. *repens* was moderately aggregated: cell clusters consisted of 10–50 cells. In the beginning of subculture cycle the culture contained both meristem- and parenchyma-like cells in equal proportions. At the end of cultivation cycle the proportion of parenchyma-like cells increased twofolds and elongated cells also appeared [14]. Cell suspension of *P. ginseng* contained small aggregates (90 % of aggregates were comprised of 5–20 cells). Majority of cells (80–90 %) in the culture were small meristem-like cells. The fraction of parenchyma-like and elongated cells was insignificant; the ratio of cell types remained constant during the cultivation period [156].

Thus the suspension cultures of the two *Panax* species differed significantly in growth pattern and cytophysiological characteristics. These differences are likely to be caused by species-specific factors and/or different hormonal composition of the media.

Dammarane Ginsenoside Content

The cell cultures of *P. ginseng* and *P. japonicus* var. *repens* differed significantly in total ginsenoside content. Overall biosynthetic capacity of the *P. japonicus* var. *repens* suspension culture was considerably higher when compared to *P. ginseng*: the average ginsenoside content in the *P. japonicus* var. *repens* and *P. ginseng* cell cultures was 3.1 and 0.04 % dry weight, respectively [143, 157]. In both the species compounds from 20 (S)-protopanaxatriol group dominated: their content was five to eight times higher than 20 (S)-protopanaxadiol group [133, 140, 143]. Similar data was obtained from cell cultures of other ginseng species (Fig. 23.6). It is also important to note that biosynthesis of ginsenosides in the *P. ginseng* cell suspension was not stable neither quantitative nor qualitative: sometimes 20 (S)-protopanaxadiol-type ginsenosides were nearly absent [143].

The effect of culture conditions, predominantly the hormone composition was studied for both the ginseng species.

The replacement of 2,4-D to α-NAA in the *P. ginseng* cell culture led to 1.5–2 fold decline in the growth rate within three-to-four cultivation cycles and increase in cell aggregation [156]. In *P. japonicus* var. *repens* cell culture the replacement of α-NAA by 2,4-D did not affect the culture growth [14, 157]. The increase in total auxins content of the medium (2,4-D and α-NAA added at 2 $mg \cdot L^{-1}$ each) resulted in low aggregation in the culture [158].

Use of α-NAA instead of 2,4-D during six to seven cultivation cycles enhanced ginsenoside accumulation in *P. ginseng* cell culture from 0.1–0.3 to 6–8 % dry weight. The increase occurred gradually: from 0.04 to 0.5 % during the first cycle and two to threefold in each of the following cycles. It is likely that such acceleration of ginsenoside production in the cell culture of *P. ginseng* was brought about by α-NAA induced cell differentiation. These processes were manifested by the increase in (i) the proportion of cell aggregates, (ii) cell volume; (iii) number of the cells with doubled nuclear DNA [14, 156, 157]. The recorded changes in cell differentiation pattern were accompanied by altered culture growth rate.

In the *P. japonicus* var. *repens* cell culture the replacement of α-NAA (the usual source of auxins for this culture) with 2,4-D did not lead to significant changes in ginsenoside production. Despite of the decline in cell aggregation, which was induced by replacement of α-NAA by 2,4-D, high level of ginsenoside accumulation (ca. 3 % of dry weight) was retained for six subculture cycles [14, 157].

Even more pronounced effect was observed when *P. japonicus* var. *repens* cell culture was grown in 10-L aerated bioreactor in semi-continuous mode. In the medium containing 2,4-D and α-NAA a decline in ginsenoside content from 3 to 0.5 % was observed at the end of the third cultivation cycle and to 0.3 % at the end of the sixth cultivation cycle. At the same time, in the culture grown on the medium

supplemented with α-NAA high ginsenoside production was retained (at least 1–2 % dry weight) [140, 158].

Malonyl-Ginsenosides in Ginseng Cell Cultures

In the past, ginsenosides of 20 (S)-protopanaxatriol-type have been considered to prevail in ginseng cell cultures. This conclusion was based fully on the detection of seven neutral ginsenosides, Rg_1, Re, Rf (the Rg-group) and Rb_1, Rc, Rb_2, Rd (the Rb-group), for which the commercial pure standards are available [129]. At the same time, the intact plant usually contains not only the free dammarane glycosides but also their esters with different aliphatic acids such as crotonic, acetic or malonic acid. The malonyl derivatives of ginsenosides are the most widespread. Several studies applied modern methods of extraction and analyses and revealed that over 50 % of total dammarane glycosides in fresh roots of *P. ginseng* and *P. quinquefolium* were comprised of malonylated ginsenosides [159, 160]. Thus, the method based on free ginsenoside content was considered to be bias and prone to errors.

The biosynthetic pathway leading to acylated, in particular, malonylated, forms of ginsenosides in cell cultures remains almost unknown. The transformed hairy root cultures of *P. ginseng* were shown to contain significant amounts of malonyl derivatives of the Rb-group ginsenosides [161, 162] though the malonyl-glycosides were not quantified. There are some data suggesting that malonylated derivatives of ginsenosides can be formed in the cell cultures of *P. ginseng* [163, 164].

Considerable amounts of malonyl-ginsenosides Rb_1 (identification was based on ^{1}H- and ^{13}C-NMR data) and Rc, Rb_2 and Rd (RP-HPLC-MS identification) were found in the cell cultures of *P. japonicus* var. *repens* [165, 166].

Variation in the content and composition of the individual ginsenosides biosynthesized in the course of 21-days growth cycle of the cell-suspension culture of *P. japonicus* var. *repens* in flasks was studied by RP-HPLC-UV analysis [166]. Seven ginsenosides were identified: Rg_1, R_0, malonyl-Rb_1, Rb_1, Rc, Rb_2 and Rd. The total amount of ginsenosides usually varied from 30 to 40 mg $\cdot$ g^{-1} dry weight, while the three ginsenosides namely Rg_1 (Rg-type), R_0 (oleanane-type) and malonyl-Rb_1 (Rb-type) accounted for 80–95 % of the total ginsenoside content. Ginsenosides Rb_1, Rc, Rb_2 and Rd (all of Rb-group) were detected at very low levels below 15 % of the total ginsenoside content. These results suggest that in cell suspension culture three ginsenosides (Rg_1, R_0, and malonyl-Rb_1) are the major storage products originating from the ginsenoside biosynthetic pathway. This finding is supported by the ginsenoside profiling data recorded over a 4-year cultivation period (Fig. 23.7).

While the total amount of the ginsenosides varied over a wide range (5.0–49.4 mg g^{-1} dry weight), the ratios of the major groups remained nearly constant: Rg_1, R_0, and malonyl-Rb_1 accounted for 75–93 % of the total ginsenoside content [166]. These findings suggested that both neutral and malonylated ginsenosides should be analyzed to determine actual ginsenoside content in cell-suspension culture.

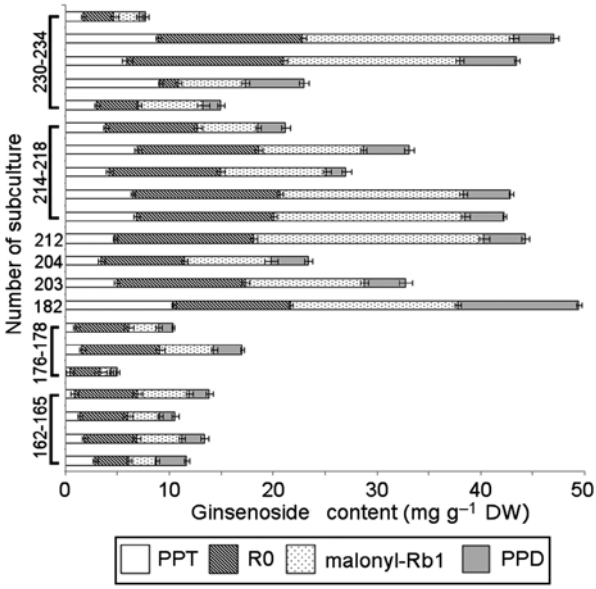

Fig. 23.7 Variations in the amounts of the major groups of ginsenosides in *P. japonicus* var. *repens* cell-suspension culture during 4 years of monitoring (21 independent growing cycles) [166]. PPT = Rg_1; PPD = $Rb_1 + Rc + Rb_2 + Rd$. Analysis was made on day 21 of a subculture

It is well known that secondary metabolic pathways are spatially associated with the compartmentalization of intermediates and end products [29, 167]. Thus, we suggested a tight coordination between ginsenoside accumulation in cell culture of *P. japonicus* var. *repens* and their sequestration into metabolically inactive cellular storage compartment such as the vacuole [168]. This suggestion also takes into account that the triterpene glycosides are a plausible substrate of various glycosidases that produce toxic progenins and aglycones [11, 84]. For example, the Rb-type ginsenosides might undergo non-specific enzymatic cleavage because the *β*-glycosidic bond at the third position of triterpenic aglycones is a common structural motif in nature [169, 170]. Our studies showed that Rb-type ginsenosides (particularly Rb_1) in suspension cell culture of *P. japonicus* var. *repens* were represented mainly by their malonylated forms. The malonylation of various glycosides is a common phenomenon in the plant kingdom [171]. Malonylated glycosides of phytohormones [172], chlorophyll catabolites [173], products of xenobiotic detoxification [174, 175] and several classes of secondary metabolites [171, 176, 177] have been found in different plant species. This modification may alter the molecular properties of parental glycosides in several ways but mainly by preventing the enzymatic degradation of the glucoconjugates and targeting them to specific compartments, such as the vacuole [178–180]. We can speculate that the malonylation of the Rb-type ginsenosides is involved in the regulation of their hydrolysis and cellular compartmentalization in *P. japonicus* var. *repens* cell-suspension culture.

On the contrary to the Rb-type ginsenosides, esterification of Rg-type ginsenosides (particularly malonylation) was not typical [124, 181]. Therefore, we suggest that the specific glycosylation of the Rg-type ginsenosides (attachment of one of the

sugar chains to the α-hydroxyl group at the C-6 position of the dammarane-type aglycone) made them resistant to non-specific glycosyl hydrolases [124, 170], enabling accumulation of ginsenoside Rg_1 without significant disturbance in the metabolic activity of *P. japonicus* var. *repens* cells *in vitro*.

Oleanolic Acid Glycoside Content

Accumulation of the significant amounts of ginsenoside R_0 is observed in suspension cell culture of *P. japonicus* var. *repens*. This finding is consistent with the literature reports [104, 120, 125] emphasizing the predominance of oleanolic acid glycosides as a characteristic trait of this species. Structurally, ginsenoside R_0 belongs to a widespread family of glycosides – glucuronide oleanane-type triterpene carboxylic acid 3,28-bidesmoside, GOTCAB in plant kingdom. These glycosides are characterized by the presence of glucuronic acid residue attached to the hydroxyl at the third position of the aglycone [182]. This structural motif is extremely resistant to non-specific hydrolysis [120]. Attachment of glucuronic acid can play a role in the distribution of metabolites between the cell compartments, for example, molecules tagged with glucuronic moiety are usually targeted to the vacuole [183, 184]. Similar mechanism could be involved in the formation of the ginsenoside R_0 of the *P. japonicus* var. *repens* cell cultures as well as intact plants of other ginseng species.

General Characteristics of TG Accumulation in Ginseng Cell Culture

Summary of TG biosynthesis and accumulation in plant cell cultures, and their differences from SG in general:

1. High ginsenoside content is not necessarily found in every ginseng cell strain. A decline in ginsenoside content occurred sometimes during prolonged cultivation or as a result of change in cultivation medium. However it is possible to obtain strains with robustly high TG content. Optimization of hormonal concentration in culture medium, treatment with stress hormones (e.g. jasmonates) and elicitors often resulted in a considerable improvement of TG production.
2. In many cases alteration in ginsenoside groups in comparison to the intact plants was recorded: in cell culture the protopanaxatriol-type ginsenosides (Rg-group) were often prevailing over the protopanaxadiol-type ginsenosides (Rb-group). The diversity of the glycosides declined in certain strains.
3. In cell culture of *P. japonicus* var. *repens* protopanaxadiol-type glycosides were mainly represented by their malonylated derivatives. This could be explained by (i) necessity of their compartmentalization (vacuole targeting), (ii) lower stability (due to glycosylation at the third C-3-position of dammarane), and (iii) higher toxicity in comparison with protopanaxatriol-type glycosides.

In general we can conclude that ginsenosides do not play a significant role in proliferation of de-differentiated *in vitro* ginseng cell and/or population. Therefore

biosynthesis and accumulation of these compounds is often unstable in cell culture and depends heavily on the cultivation conditions (medium composition, signal molecules, stress, etc.) and physiological state of the population (stages of growth and differentiation).

On the contrary high ginsenoside content observed in certain cell cultures for a long time (e.g. 15 years at the level of 0.5–5 % of dry weight in the case of *P. japonicus* var. *repens* cell cultures) suggests that ginsenosides are essential for cell growth *in vitro*. From this standpoint ginsenosides with different structural groups are not equal. The predominance of the Rg-group ginsenosides frequently observed *in vitro* could suggest they either play a special role in cells *in vitro* or, at least, do not affect their metabolism. Low and unsteady content of neutral Rb-group ginsenosides indicates less significant, if any, role of these compounds in the cell growth *in vitro*. Another possible reason is high toxicity of the Rb-group ginsenosides and/or products of their hydrolysis for vigorously proliferating cells, because carbohydrate moiety at C-3 position of the aglycone of Rb-group can be cleaved off, unlike the carbohydrate chain at C-6 of Rg-group, by non-specific β-glycosidases resulting in the formation of the toxic prosapogenins. Malonylation of the Rb-group alters their properties and targets them to the vacuole. The same is probably true for glucuronide derivatives of oleanolic acid.

Thus, we suggest that ginsenoside biosynthesis in cell culture is closely associated with their compartmentalization which depends on the molecule structure. In particular, the accumulation of the 20 (S)-protopanaxadiol-type ginsenosides depends on malonylation, which likely targets them to the vacuole. This finding provides a new understanding of the ginsenoside accumulation and may help with the rational optimisation of their production in various ginseng cell cultures.

Triterpene Glycosides in the Cell Culture of *Polyscias*

Apart from different ginseng species other representatives of Araliaceae family were introduced to the *in vitro* culture. Recent studies described the *in vitro* cell cultures of different members of the genus *Polyscias*. These plants are broadly used in traditional medicine in South-East Asia. For example, *Polyscias filicifolia* is included in the official Vietnamese pharmacopoeia as an anti-fatigue and cardiac drug [185]. *P. filicifolia*, a relative of *P. fruticosa* is also used for its anti-fatigue, roborant, immune-modifying and anti-dizziness effects [186], but *P. fruticosa* is not as much studied as *P. filicifolia*.

Triterpene glycosides are the essential secondary metabolites of the genus *Polyscias* as well as the other representatives of Araliaceae family. Till date, composition of triterpene glycosides was studied only in 6 out of 130 members of *Polyscias*: *P. scutellaria, P. fruticosa, P. amplifolia, P. guilfoylei, P. fulva* and *P. dichroostachya* [187–190]. All studied species contain triterpene glycosides of the oleanane series.

Studies on cell and tissue culture of *Polyscias* species were initiated in the USSR in the beginning of 1970s. First callus cultures of *P. filicifolia* and *P. balfouriana* were obtained in 1971–1975 [191]. Preliminary phytochemical analysis of the

P. filicifolia callus cultures revealed the presence of significant amounts of starch, free amino acids, reducing sugars, sitosterol and triterpene saponins [192]. The maximum content of the 'saponin fraction' (5.8 %) was recorded on the 5th and 25th day of subculture cycle that corresponded with the peaks of mitotic activity [192]. It should be emphasized that exact structure of the discovered glycosides was not determined; i.e. only 'total glycoside fraction' ('saponin fraction') was studied in these works.

At the end of last century the strain BFT-001-95 of *Polyscias filicifolia* callus and suspension culture was obtained. These cultures were grown in different systems and under different conditions: in flasks (batch mode) and in laboratory-scale bioreactors (continuous and semi-continuous modes). The up-scaled cultivation of *P. filicifolia* cell suspension was performed in the industrial 630 L bioreactor [193, 194]. The analysis of biological activities of the *P. filicifolia* cell culture biomass obtained under diverse cultivation conditions was also performed [195].

Now the cell culture of *P. filicifolia* is used in production of bioactive food additive "Vitagamal" [193, 194]. The authors related bioactivity of the *P. filicifolia* cell cultures to the presence of the triterpene glycosides from the oleanane series in its biomass. The potential presence of triterpene glycosides in the biomass of this suspension culture was studied after nearly 20 years the cell culture was induced. The attempt to find triterpene glycosides or aglycones (of oleanolic acid in particular) in the biomass was unsuccessful [196]. This suggested that high biological activity of *P. filicifolia* cell biomass could be related to other compounds such as polyacetylenes. In 2005, a new line of *P. filicifolia* and – for the first time – callus and suspension culture of *P. fruticosa* [186] were obtained. The new line of *P. filicifolia* suspension cell culture contained almost complete spectrum of oleanolic acid glycosides typical for intact plants. It should be pointed out that this suspension cell cultures was obtained directly from leaves bypassing the stage of callus culture [186].

Triterpene glycosides of oleanolic acid were also found in the suspension cell culture of *P. fruticosa* [197, 198]. Chromatographic (TLC and HPLC) analyses revealed that the major triterpene glycosides of *P. fruticosa* suspension cells are identical to those isolated from leaves of *P. filicifolia*. The major components in the *P. fruticosa* cell cultures were polyscioside E, 28-*O*-*β*-D-glucopyranosyl ester of 3-*O*-*β*-*d*-glucopyranosyl-(1 → 4)-*β*-D-glucuronopyranoside of oleanolic acid (named Pol 3) and ladyginoside A.

To reveal the patterns of individual triterpene glycoside formation in the cell culture of *P. fruticosa* changes in the content of these compounds were studied during a cultivation cycle in flasks. It was found that the total glycoside content increased during the whole cultivation cycle. The maximum glycoside content (0.5 % of dry weight) was recorded in the end of exponential growth phase (14 days). Same was observed for suspension cell culture of *Cyclocarya paliurus* (Juglandaceae), in which the highest accumulation of triterpene acids (oleanolic and ursolic) took place between the end of exponential and the beginning of stationary phase of the culture growth [199].

All found glycosides (polyscioside A, Pol 3, polyscioside E and ladyginoside A) were present in *P. fruticosa* cell culture during the whole cultivation cycle. The major components were polyscioside E and ladyginoside A comprising, respectively, 10–40 and 40–70 % of the total glycosides depending on cultivation period. Of special interest is the finding of significant amounts of monodesmoside ladyginoside A, the most hydrophobic of the glycosides. Its accumulation sustained over the whole cultivation cycle. It is well known that accumulation of monodesmosides is not usually observed in leaves and roots of intact Araliaceae plants, which synthesize saponins of oleanane series [120]. Thus, the biosynthesis of monodesmoside might be unique for *P. fruticosa* and/or can be the result of tissue culture conditions. Additional research is needed to confirm this suggestion.

In general we can conclude that the pattern of triterpene glycoside formation by cell culture strains of two *Panax* and two *Polyscias* species implements the second strategy described under "***Principle 2***" of Sect. 23.1.

23.4 Diterpenoids in Plant Cell Cultures

23.4.1 Taxoids in Cell Cultures of Taxus spp.

Taxol, or paclitaxel, a complex diterpenoid from the bark of yew tree (*Taxus* spp.) is probably the most promising anti-tumor agent of plant origin. Its biological activity is attributed to the unique effect of stabilizing cell microtubules [200]. The evergreen trees and shrubs of *Taxus spp.* are the main natural source of taxan diterpenoids, a group of active molecules of the same pentamethyl tricyclopentadecan structure. However, slow growth of the majority of yew species and relatively low paclitaxel content in the bark and leaves of the plants (0.01 and 0.035 %, respectively) made paclitaxel production from natural sources both time-consuming and economically ineffective [201]. Hence it is important to find the alternative sources of taxol for pharmacological industry.

Since 1997 the Atlantic Forestry Centre of Canadian Forest Service has been engaged in a program for developing ecologically sustainable harvesting protocols of yews in natural stands converting elite cultivars of the wild species into a commercially reared crop [202]. Similarly the Yewcare Company began to plant *T. chinensis* in the nature reserve of Da Huan Mountain in the province of Yunan (China) in 2004. This Taxus plantation covers now more than 30 km^2 and is the largest yew tree provider in the world (http://www.yewcare.com/index.ph.) Another alternative source is semi-synthetic production of taxol, which utilises intermediates such as baccatin III and 10-deacetylbaccatin III, found in needles of *Taxus*. BMS, a leading global supplier of taxol, has a farm of 30 billion yews to supply the bark and needles necessary for the extraction of intermediates [203]. In 2007, Indena developed and patented a protocol of taxol semisynthesis based on 10-deacetylbaccatin III, which is extracted from *T. baccata* trees cultivated in the company plantations (www.Indena.com).

Another possible alternative and environmentally sustainable source of taxol and analogue compounds is plant cell culture. At present it is possibly the most actively developing area of biotechnology [204]. The first report on callus induction and proliferation from gametophytes of *T. baccata* was published in 1973 by Rohr [205]. Further studies have been primarily focused on optimization of culture conditions for better cell growth and taxol production. Within the past 40 years a number of independent research groups performed a broad screening of treatments to increase taxol production in the cell cultures. Different strategies have been applied such as optimization of culture conditions, selection of high-producing cell lines and addition of elicitors and precursors (for the latest reviews [206, 207]). Based on the developed cell lines commercial production of taxoids has been established in many countries. At present Python Biotech is the largest producer of paclitaxel *via* plant tissue culture employing a large-scale bioreactor with a capacity of up to 75,000 L [208]. Another company, Korean Samyang Genex, uses Taxus plant cell cultures to produce paclitaxel under the brand name of Genexol® (http://www.genex.co.kr/Eng/).

On account of a large volume of information available, in this chapter we present a brief overview of the main principles underlying taxoid biosynthesis and its regulation in yew cell cultures *in vitro*.

1. Cell cultures, both callus and suspension, have been obtained so far: *Taxus baccata, T. brevifolia, T. cuspidata, T. canadensis, T. media, T. wallichiana, T. andreanae and T. mairei.* These cultures were reported to be slow growing, which is common feature of cultures of coniferous origin. However, their growth could be speeded-up by optimizing the composition of culture medium or selection methods.
2. The vast majority of cell cultures produced negligible amounts of taxoids, 0.0001–0.01 % dry weight, or no detectable taxoids.
3. Taxoid content in cell cultures exponentially increased under certain treatments. The following treatments were found to be the most effective:

   (a) Selection of the most productive cell lines;
   (b) Two-step cultivation. At first, culture conditions should be optimized to increase biomass production. In the second step, taxol accumulation should be stimulated by changing osmotic potential and growth regulator composition in culture medium.
   (c) Application of elicitors and stress-related hormones to induce taxoid production. Jasmonates (JA and MeJA) were found to be the most effective.
   (d) Paclitaxel is normally accumulated in vacuoles and apoplast of cultured cells. In contrast to triterpenoids, considerable amounts of paclitaxel have been detected in culture medium, presumably caused by its diffusion from apoplast. As a result the amount of paclitaxel in cells could vary from 30 to 100 % depending on the diffusion rate which is found to be species-specific and dependent on culture conditions.
   (e) In gel cell immobilization with or without subsequent MeJA treatment.

4. Metabolic engineering targeting the genes encoding taxadiene synthase, 10-deacetylbaccatin III-10-O-acetyltransferase (dbat) and 3-N-debenzoyl-2-deoxytaxol-N-benzoyltransferase (dbtnbt) has been reported. So far this method had no significant effect on the taxoid production.

Based on the information discussed above and available literature we assumed that the major principles of taxoid production in cultured plant cells differed from those of triterpenoids:

1. Different pathways of biosynthesis. Taxoids are diterpenoid molecules of complex structure, and the first stages of their biosynthesis can be performed *via* plastid-localized MEP-pathway. To support this idea Eisenreich et al. [209] showed that IPP involved in the biosynthesis of the taxane ring was formed *via* the MEP pathway. However, other studies [28] demonstrated the involvement of the cytosolic pathway. A recent study of *T. baccata* cell cultures showed that while taxol biosynthesis was blocked by the addition of fosmidomycin, an inhibitor of the plastid pathway, it was also reduced by mevinolin, an inhibitor of the cytosolic pathway, indicating that both pathways could be involved [210]. Thus, plastids play an important role in taxoid biosynthesis, although their number in cultured cells is scarce.
2. Complexity of biosynthesis localization. While the first steps of taxol biosynthesis are likely to be performed in plastids, a number of the following stages might occur in different compartments of cytosol. For instance, the enzyme cytochrome P450 taxadiene-5-hydroxylase (T5-H), which catalyses hydroxylation at the C-5 position of the taxane ring, is a protein of 56 kDa with an N-terminal of membrane translocation sequence targeting it to the endoplasmic reticulum. A key enzyme of the following stage, a specific taxadiene-5α-ol-O-acetyl transferase (TDAT), is a protein of 50 kDa that bears no N-terminal organellar targeting information [25]. Final products such as paclitaxel and baccatin III are then transported to vacuoles and/or periplasmic space involving yet unknown mechanisms.
3. Fork-branched biosynthesis. Paclitaxel formation involves 19 steps catalyzed by specific enzymes. One of the intermediate products, taxa-4(20),11(12)-dien-5-ol, which serves as a substrate for TDAT, can be also involved in a side reaction catalyzed by Cyt P450-dependent hydroxylase, taxadiene-13α-hydroxylase, yielding taxa-4(20),11(12)-dien-5-13-diol [204]. It was found that this alternative step is especially active in cell cultures elicited with methyl jasmonate [211]. This fact shows that taxol biosynthesis is not a linear pathway and includes branch points, which can lead to other taxoids.
4. Properties of the biosynthate. Taxoids are hydrophobic and toxic molecules. Paclitaxel was involved in apoptosis of *Taxus cuspidata* cell suspension [212]. Correlation between paclitaxel accumulation and increase in number of dead cells was reported for cell cultures of other *Taxus* species [213, 214].

23.4.2 Steviol Glycoside Formation in Cell Culture of Stevia spp.

Steviol glycosides (StG) are the group of molecules incorporating steviol, the tetracyclic diterpenoid of *ent*-kaurane type, as an aglycone. Many compounds of this group are 100–400 times sweeter than sucrose, but low in calories, non-toxic, non-mutagenic and are hardly assimilated by human body [8]. Due to their hypoglycemic effect StG are very promising as sweeteners in the diet of patients with dysfunctions of carbohydrate metabolism, especially for those with diabetes [8].

Steviol glycosides are found in large amounts in leaves of *Stevia rebaudiana* Bertoni, Asteraceae, a perennial shrub native to Northeast Paraguay. Three other species: the Mexican *Stevia phlebophylla* A. Gray, the Chinese blackberry *Rubus suavissimus* S. Lee (Rosaceae) and the Japanese perennial *Angelica keiskei* (Miq.) Koidz. (Apiaceae) also contain steviol glycosides. Thirty four steviol glycosides have been identified in *S. rebaudiana* together with other eight oxidized steviols, including isomers and glycosides.

Stevia cell cultures were obtained by several research teams [215–220]. Most of these cultures demonstrated stable and intensive growth but lacked StG production. The only exception was reported 30 years ago: the callus culture originated from leaf blade showed steviol content as high as 16 % dry weight after 70 days of cultivation [216].

Apart from *S. rebaudiana,* callus cultures of *R. suavissimus* were also obtained with modest rubusoside content, which was promoted by blue light after 28 days in the dark [8]. However, neither blue nor red or white light promoted StG formation in *Stevia rebaudiana* cell cultures.

It was shown that StG content of cultivated *Stevia* plants is five to ten times lower than that of intact plants [8, 221, 222].

Compared to the other cell cultures obtained from, for example, *Taxus spp.*, the literature considering StG formation in cell cultures is rather scarce and fragmented. Therefore it is difficult to generalize on the patterns of StG formation in cells cultured *in vitro*.

In authors' laboratories StG formation was studied in 12 *S. rebaudiana* genotypes originated from Russia, Brazil, Paraguay and Japan. These genotypes had ploidy level from 2n to 5n. Cell cultures were induced from all genotypes and analyzed on StG production. StG content declined in a range from 'outdoor plants (30–80 mg g^{-1} dry weight) to greenhouse plants (15–25 mg g^{-1} dry weight) and finally plants grown *in vitro* (1–6 mg g^{-1} dry weight) [15, 223, 224].

More than 20 callus and suspension lines were obtained from the plants of most productive genotypes and their growth and biosynthetic profiles have been investigated. Both genotype and explant type were found to influence profoundly the morphological and physiological traits of the callus cultures. The ability to produce small amounts of StG (steviol, rebaudioside B, stevioside) was detected in the callus cultures derived from only one genotype. However, even in this cell line, the ability

to produce StG was lost completely after 2-year cultivation. Other callus cultures contained StG only in trace amounts [15, 223, 224].

Growth characteristics of callus cultures sustained upon transition to submerged cultivation, i.e. suspension culture. Although in suspension cultures higher StG formation compared to callus cultures was recorded, yet StG content remained very low (20–120 μg g^{-1} dry weight; productivity of 0.1–0.8 mg L^{-1}). The major glycoside in all cultures was stevioside. Rebaudoside A was absent in some strains. Rebaudiosides C and B and steviolbioside were found only in trace amounts. Optimization of the cultivation conditions on carbohydrate sources including substitution of sucrose to fructose, glucose, maltose, galactose, arabinose, raffinose, rhamnose and sorbitol and changes in sucrose content from 2 to 5 % did not accelerate StG formation. Optimization of mineral salt concentration was turned to be similarly inefficient [15, 223, 224].

Thus, heterotrophic cell cultures of *Stevia rebaudiana* lacked StG completely or produced lower StG amount when compared to intact plants or plants cultured *in vitro*; the diversity of StG also decreased.

Since StG are synthesized *via* MEP pathway and predominantly localized in leaves an effort has been made to obtain mixotrophic cell cultures. Cultivation of callus cultures under illumination (2,000 lx) for several cycles led to the formation of numerous chloroplasts. Light effected callus cultures differently: some cultures intensified growth, some did not show any change. Fortunately in several cultures, in which growth was promoted by light, StG accumulation was also enhanced with its content reaching 30–60 μg g^{-1} dry weight. The composition of StG was strain-dependent with stevioside and rebaudioside A and C being the major components [15, 223, 224].

Organogenesis (gemmogenesis) was induced in several mixotroph callus cultures, and appearance of morphogenic structure and shoot formation led to a considerable intensification of StG biosynthesis. The de-differentiated cells of morphogenic callus contained 70–90 μg · g dry weight StG whereas shoots formed from callus contained tenfold higher amount up to 0.6 mg · g^{-1} StG. The latter was 30 % of StG content of the donor plants *in vitro* [222, 224]. The content of StG in cultured cells and different organs of *S. rebaudiana* is summarized in Table 23.8.

We conclude that effective production of StG in heterotrophic cell cultures was not achieved *via* any tested biotechnological method. Chloroplast formation and/or organogenesis were prerequisite for acceleration of StG biosynthesis. StG production increases as the plant development progresses (compared to greenhouse and *in vitro* plants).

Analysis of the patterns of StG formation in cell cultures demonstrates the following tendencies:

1. Production of the target compound depends not only on the presence of the organelles (plastids), but also on their specialization (must be chloroplasts). In plants StG are synthesized only in green leaves, but not in roots and other heterotrophic organs/tissues because only leaves contain sufficient amount of chloroplasts and certain enzymes for StG formation [8].

Table 23.8 Steviol glycoside (StG) content of *S. rebaudiana* plants and *in vitro* cultures ($mg \cdot g^{-1}$ dry weight) (CV < 30 %) (According to [15, 223, 224])

Culture	Sample	Stevioside	Rebaudioside A	Rebaudioside C	Total StG
Intact plants (2 *n*)	Leaves	24.9	12.0	4.6	41.5
	Stalks	4.5	2.6	0.4	7.5
Plants *in vitro* (2 *n*)	Leaves	3.3	1.91	0.7	5.9
	Stalks	0.8	0.6	0.1	1.5
Etiolated shoots *in vitro* (2 *n*)	Shoots	0.28	0.18	0.07	0.5
Green morphogenic callus[a]	Shoots	0.39	0.11	0.05	0.6
	Cells	0.07	0.02	0	0.09
Mixotrophic[a] callus	Cells	(0.03)	0.02	(0.02)	0.05
Heterotrophic[a] cell suspension	Cells	0.09	(0.01)	0	0.09
Heterotrophic[a] callus	Cells	Traces	0	0	Traces

[a]Data obtained from several cell cultures; compounds, which were not always present are taken in brackets

2. StG and gibberellins in plant leaves are formed from the same precursor. There are evidences [8] suggesting that switching biosynthesis from gibberellins to StG is not due to competition for substrate but leaf development stage. In particular, the enzymes catalyzing StG biosynthesis are vigorously worked in senescing leaves and the enzymes of gibberellin biosynthesis in young leaves. Therefore, temporal 'separation' plays an important role in the regulation of StG biosynthesis.
3. Localization of StG biosynthetic stages could be even more complex, than that of taxoids. It could involve not only cellular compartments, but also specialized morphological structures. There are indications that special leaf glandules are involved in StG formation and storage.

In general we assume that formation of StG in cell cultures of *S. rebaudiana*, similar to taxoid formation, proceeds as outlined in ***Principle 3*** of Sect. 23.1. Still this case is more difficult since no significant StG production have been achieved in de-differentiated cells so far. Formation of morphological structures proved to be necessary for the fully functional StG biosynthesis pathway.

23.5 Biotechnological Aspects of Isoprenoid Production from Plant Cell Cultures

The industrial use of plant cell culture presumes cultivation in large bioreactors up to 75,000 L [208], that imposes a number of prerequisites [225, 226] to support the highest possible culture growth and accumulation of a target compound.

The following traits of plant cell culture should be considered when large-scale cultivation is sought [7, 30, 227]:

- Sensitivity of large cells with vacuole(s) to shear stress caused by mechanical stirring which is thought to be related to fragility of cell walls.
- Rapid increase in sedimentation rate due to cell aggregation and increase in cell suspension viscosity at the end of cultivation cycle often require optimization of stirring process.
- Foamy and highly adhesive above culture broth (so-called 'meringue' formation).
- High importance of aseptic environment, because antibiotics cannot be used and extended batch cycle (ranges from 5 to 20 days to 6 months) under continuous or semi-continuous cultivation.

According to recent reports [31, 228], the adverse effects of shear stress caused by stirring are considerably overestimated in the case of plant cell cultures.

Many of the difficulties that in the past hindered the industrial cultivation of plant cells in bioreactors are successfully resolved [7, 30, 227]. Up-scaling is now technological rather than scientific task. Use of plant cell cultures for industrial production of secondary metabolites has been reviewed, but only a few examples of successful commercial process could be found [7]. Obviously, the main reasons are high cost and demanding technological requirements. To increase the efficiency cell lines with elevated productivity are required as well as innovative methods of biomass production.

Productivity of the cell cultures can be increased by optimization of cultivation conditions. Continuous cultivation in bioreactors is a powerful tool to burst biomass production. Unfortunately, it proved to be unsuitable for commercial cultivation of plant cells.

An attractive alternative is semi-continuous cultivation. This method was implemented in our laboratory using bioreactors with the working volumes ranging from 15 to 550 L and equipped with different stirring systems.

Regardless of the bioreactor capacity the new medium was supplied at the slow-down stage with simultaneous off take of culture. The suspension was diluted with fresh medium to the level which allowed bypassing a lag phase (2.0–4.0 g dry weight per L). The optimal starting density was different for different cultures. The growth curves for the final bioreactor (630 L) are shown in Fig. 23.8. It was shown that plant suspension cultures of *D. deltoidea, Polyscias filicifolia* and *Panax japonicus* retained satisfactory growth and biosynthetic characteristics upon transition to prolonged semi-continuous cultivation in bioreactor. Thus, we concluded that the proposed up-scale scheme is suitable for industrial cultivation.

It is also important that the scheme is universal and may be optimized for different cultures within two or three steps. For this optimization it is necessary to determine the minimal inoculum amount for different cultures to eliminate the lag phase and achieve optimal cycle duration. The biomass should be harvested at the very end of the exponential phase.

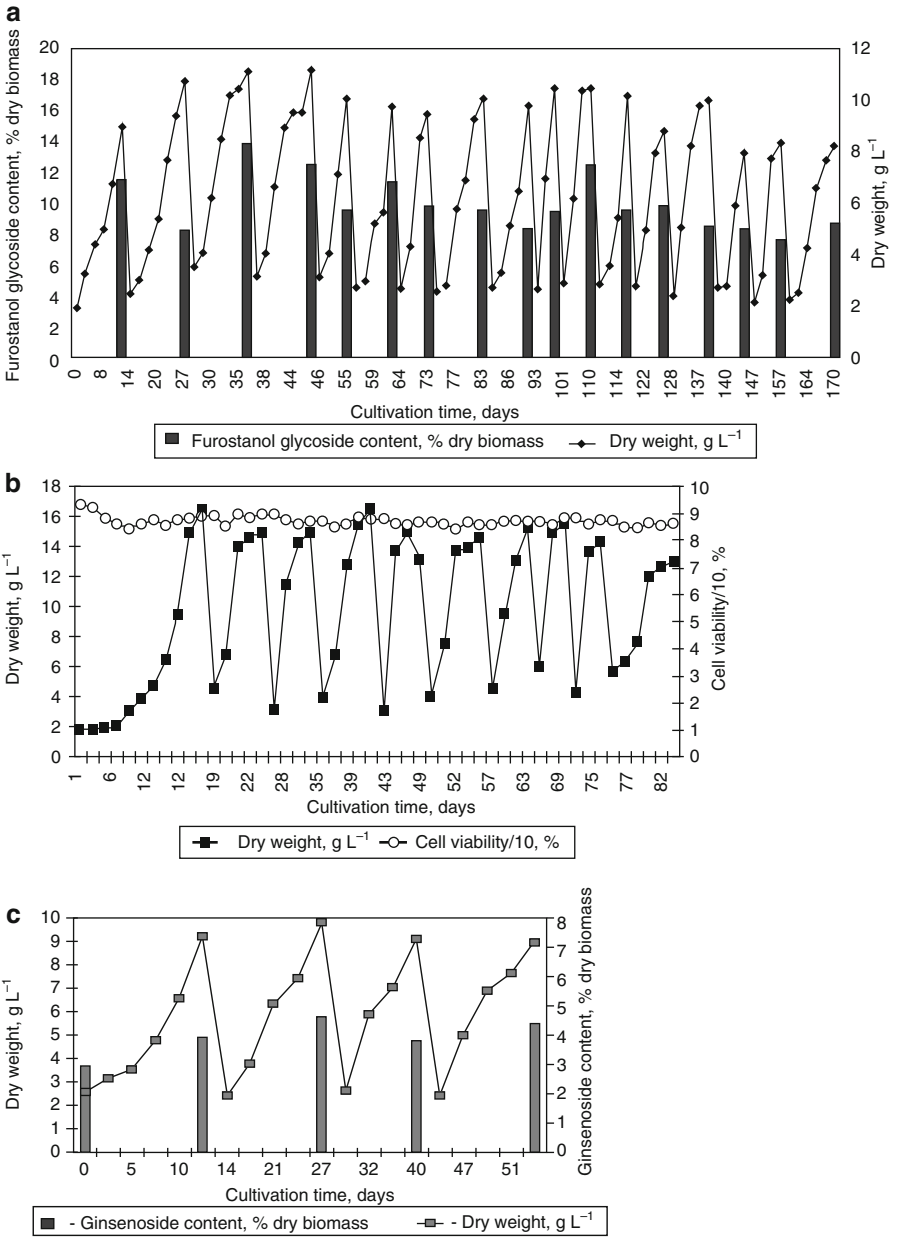

Fig. 23.8 (**a**–**c**) Plant cell cultures growth and secondary metabolites accumulation in 630-L bioreactors, operated in semi-continuous mode. (**a**) *Dioscorea deltoidea.* (**b**) *Polyscias filicifolia.* (**c**) *Panax japonicus* var. *repens*

During such up-scaling both DM-0.5 strain of *D. deltoidea* and *P. japonicus* cell culture retained the capability of sustained production of furostanol glycosides and ginsenosides, respectively, in the amount sufficient for the industrial production.

Generally, the efficiency of biomass production increased by 15–20 % under semi-continuous cultivation due to the absence of lag phase and the technical maintenance in-between-cycles. Hence, semi-continuous mode shall be considered as optimal for the industrial production of plant cell culture biomass.

23.6 Cryopreservation of Plant Cell Cultures with Enhanced Isoprenoid Production

Storage of cell cultures at cryogenic temperatures, below −130 °C, eliminates repetitive subcultures thus reducing the risks of culture loss caused by contamination or technical errors. It also decreases the rate of genetic and epigenetic variations, prevents the loss of regeneration potential and changes in secondary metabolite profile [229, 230].

Building on the classical works of Quatrano [231] and Latta [232], successful cryopreservation has been reported for cell cultures of various medicinal plants which can be potentially used for the production of valuable secondary metabolites. This includes *Digitalis spp*. [233, 234], *Rhaponticum carthamoides* [235], *Artemisia annua* [236], Ginkgo *biloba* [237, 238], and many others (for the most recent reviews see [229, 239–241]).

The majority of cryopreservation protocols resulted from the empirical approaches [242] or have been modified from those applied to cultured animal cells [242, 243]. A classical method of slow, or programmed, freezing was the most applicable so far. With *Panax ginseng* cells suspension, this method resulted in 34–51 % recovery after cryopreservation depending on preliminary treatment [131, 244, 245]. Preculture of *P. ginseng* cells in medium with high sucrose concentrations combined with 3-weeks cold-hardening improved their survival after cryopreservation when compared to the cells grown under standard conditions, assumably due to alteration in intracellular sugar content [131, 245]. Another successful protocol involved pretreatment of *P. ginseng* cell culture on medium supplemented with 20 % sucrose while the temperature of cultivation was gradually reduced from 25 to 4 °C. This pretreatment resulted in 40 % post-freeze cell viability [244]. The maximal regrowth of *P. ginseng* cells after cryopreservation was achieved using 20 % sucrose as a cryoprotector [131, 244]. Combination of 10 % glycerol and 10 % sucrose was found to be less effective [245]. Suspension cultures of *P. ginseng* and *P. quinquefolius* have been cryopreserved by programmed freezing using glycerol and sucrose as cryoprotectors [243]. Maximum post-cryopreservation viability of 55 % was observed for *P. quinquefolius* cells cryoprotected with a combination of 15 % glycerol and 10 % sucrose while pretreatment with 20 % sucrose was detrimental for post-thaw survival. Cell culture of *Polyscias filicifolia* cryopreserved by the same method regenerated at the rate of

45 % [193]. Interestingly, variation in temperature and the way of cryoprotector treatment (at once or with gradual increase of concentration) showed no significant differences in cell viability after cryopreservation.

Maximum viability of 40 % has been reported in *Taxus chinensis* cell culture cryopreserved by slow freezing method using a mixture of 0.5 M DMSO and 0.5 M glycerol [246]. Successful cryopreservation of *Dioscorea deltoidea* cell suspension *via* slow freezing method was accomplished following preculture with 0.02 M asparagine and 0.05 M alanine which induced accumulation of cells with high osmotic- and cryotolerance in the population [131, 247]. Slow-freezing method using 7 % DMSO as cryoprotector was the most effective for cryopreservation of two *D. deltoidea* cell lines [68]. The authors also showed that haploid and polyploid cells of *D. deltoidea* were more sensitive to cryopreservation-induced injuries than di- and triploid cells [68]. Cells of both *P. ginseng* and *D. deltoidea* were found to be more susceptible to cryopreservation at the beginning of the exponential growth phase [246].

Alternative approach to cell cryopreservation was reported by Joshi and Teng [248]. In their study, cells of *Panax ginseng* were exposed to gradually increasing the concentration of glycerol and sucrose followed by direct immersion in liquid nitrogen. The highest viability after cryopreservation achieved by this method was 86.5 %.

Cryopreservation procedure normally involves pretreatment of plant cells with osmotically active and/or toxic chemicals which cause plasmolysis and induce severe and often unrecoverable damages in cell protoplasts. Therefore, the retention of main growth and biosynthetic traits as well as cytological and genetic stability of cell cultures regenerated after cryopreservation should be carefully assessed. It is important that cryopreservation had no effect on ginsenoside profile of *Panax ginseng* cell cultures [244, 249]. Moreover, cultures regenerated after cryopreservation demonstrated higher maximum growth, biomass productivity and yield when compared to non-frozen cells [248]. In contrast, lower accumulation of dry cell weight was recorded for cryopreserved *Taxus chinensis* cell culture when compared with the untreated control in the course of 40-day cultivation, however, paclitaxel production was retained at the same level [246]. Diosgenine, sitosterol and stigmasterols content remained unchanged after cryopreservation of *Dioscorea deltoidea* cell cultures [131]. The same profiles of the relative DNA content have been recorded for cell cultures of *Ginkgo biloba* in the course of 24-month cultivation followed by cryopreservation [237].

Cell culture of *Polyscias filicifolia* showed 25–40 % survival after 5 years of cryogenic storage [193]. This survival was sufficient for regeneration of cell culture following a few consequent steps such as proliferation of callus on solid medium, multiplication of cell suspension in flasks and finally biomass production in semi-continuous mode bioreactors of different volumes, 15 up to 550 L. The main growth and biosynthetic traits of the regenerated culture were retained at levels comparable to those of the initial cell culture (before cryopreservation), regardless of bioreactor volume and type [193]. It is worth to be noticed that the regenerated culture retained the ability for being up-scaled to bioreactors of industrial volume. The same cell

line which has been maintained for 5 years by means of repetitive subcultures showed twofold decrease in productivity when compared to initial cell line and the culture regenerated after 5-year cryogenic storage. To our knowledge, this is the first report of bioreactor cultivation of undifferentiated plant cells after long-term cryopreservation.

23.7 Conclusions

23.7.1 Cell Cultures In Vitro as a Source of Secondary Metabolites and Associated Problems

Formation of secondary metabolites and, in particular, isoprenoids, in cell cultures *in vitro* differs significantly from that in intact plant. The difference resulted from cell dedifferentiation and continuous proliferation and also selection mechanisms being active in cell population. Physiological roles of secondary metabolites in intact plant suggest that their substantial accumulation in cell *in vitro* expected to be exceptional rather than common. Nevertheless, intense biosynthesis of a number of secondary metabolites by cells *in vitro* was reported due to (i) the diversity of functions of secondary metabolites and variety in their effects; (ii) variability of plant cells and their adaptability to *in vitro* conditions and (iii) array of methods available for culture manipulation and stimulation of metabolite production.

Study of secondary metabolism in plant cell cultures are of both fundamental and practical importance. Till date, induction and selection of cell lines with enhanced production of a target metabolite was based on empirical approach, such as optimization of culture conditions and various methods of increasing culture productivity. A profound analysis of the formation of secondary metabolites in cell cultures can make optimization process more efficient and predictable. Since the majority of secondary metabolites are not crucial for vigorous cell growth their biosynthesis in dedifferentiated cell cultures may be inhibited following a peculiar "hierarchies of arrests" which can be of chemical, biochemical and physiological origin and are summarized below:

1. Hierarchy of chemical arrests depends on possible toxic/beneficial effect of compounds and can be visualized as follows: toxic → neutral → beneficial effect on cell growth → stimulation of cell growth and proliferation. Other properties of the compound such as hydrophobicity/hydrophilicity, presence of functional groups, etc. are also important.
2. Hierarchy of biochemical arrests depends on the length and complexity of the biosynthetic pathway. Metabolites with short and unbranched biosynthetic pathway may be easier to obtain than molecules yielded from several unrelated reactions.
3. Hierarchy of physiological arrests depends on compartmentalization and temporal organization of biosynthesis. The most desirable yet the rarest option is

co-localization of both synthesis and storage in one compartment in a single cell. More often biosynthesis and storage occur in two different compartments. The worst case scenario is localization of biosynthesis in different compartments and at a certain stage of plants ontogenesis.

Depending on the position of a target compound in the hierarchy it is possible to predict its probable formation in plant cell culture and even optimal methods of treatment to increase its production. In the worst case scenario (a compound is toxic, biosynthesized in several compartments at a certain stage of morphogenesis) biosynthesis in cultures *in vitro* has not been achieved, e.g. alkaloids of morphine or dimeric indole types. Among isoprenoids diterpenoids including steviol glycosides have the most complicated biosynthesis. Furostanol glycosides have the simplest one.

To stimulate production of "difficult" compounds in plant cell cultures it is helpful to find its precursors and choose the most favorable candidate for the semi-synthesis of a final product, e.g. baccatine III for taxoids and vincamine and catharanthine for dimeric indole alkaloids. Also formation of such compounds in cell cultures could be achieved by mutagenesis and selection of the most productive cells/lines *in vitro*.

23.7.2 Population Engineering Versus Metabolic Engineering

At present two methodologies of secondary metabolite production are widely discussed in the literature: the traditional 'empirical' and the new 'rationalized' approaches. The traditional approach is based on the 'black box' strategy. It involves all manipulations described above: cell selection, optimization of the medium composition, elicitation, use of the biosynthesis precursors, cell immobilization, etc.

The 'rationalized' approach is based on changes in cell metabolism *via* the methods of molecular biology. During the last decade a considerable progress has been made in the study of genes and enzymes involved in secondary metabolism resulting in obtaining the corresponding cDNA [250, 251]. Also the role of transcription factors, promoter and enhancer regions in the regulation of the genes involved in secondary metabolism was revealed [252, 253]. Thus, the novel strategy is based on overexpression or silencing of certain genes involved in biosynthesis/regulation of production of secondary metabolites. Similar approaches are employed, apart from regulation of plant cell culture productivity, to intact plants, plant organ cultures, and, recently, to microbial cultures producing plant-specific compounds [31].

It is accepted that the 'rationalized' approach is more efficient and it should eventually replace the 'empirical' approach.

However, eukaryotic cell, especially plant cell has an array of countermeasures enabling the silencing of foreign genes and segregation of their products. Thus, the immediate success of the molecular approach is questionable, especially if we also take into account the imperfection of the current gene engineering methods and,

most importantly, vast complexity of the problem. It is not enough to obtain the expression of a single gene as such. One has to construct a pipeline of a dozen or more genes including the supply of substrate as well as translocation and compartmentalization of the product, ensuring the temporal coherence of the whole process. It is also not clear whether it is enough to achieve the expression of the biosynthesis pathway genes and/or the regulatory genes and/or transporter genes?

It is also important to note that at present the traditional approach becomes less empirical due to profound knowledge on cell life *in vitro* and peculiarities of the secondary metabolism in such cells. It can be designated as knowledge-based 'population engineering' that harnesses the control over the living and developing of population of somatic cells *in vitro*. The goals of 'population engineering' include creation of new cell populations or conditioning the existing populations to facilitate the production of a target compound. We demonstrated that it is possible to achieve this goal using high variability and adaptability of plant cell populations *in vitro* and a vast array of stimuli affecting these populations in this chapter. Further research is needed to choose the more efficient of the two approaches though we think that the optimal solution is a 'smart' combination of both.

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