Chapter 20
Strategies for Enhanced Production of Plant Secondary Metabolites from Cell and Organ Cultures

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Abstract Plant cell and organ cultures have emerged as a potential source of secondary metabolites which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colouring agents, pesticides and food additives. Various strategies have been developed over past decades for biomass accumulation and synthesis of valuable compounds. Biosynthesis of secondary metabolites are generally not directly associated with cell growth. For the enhanced production of secondary metabolites, selection of high-yielding cell or organ clones, optimization of medium and physical factors which regulate the growth and accumulation of biomass are usually done at first, then in the secondary metabolite production stage, various strategies such as elicitation, precursor feeding, replenishment of nutrients are conducted. Permeabilization and immobilization are also proved to be important for the biosynthesis of secondary metabolites in some cases. By these strategies, it is possible to produce enormous biomass with improved accumulation of secondary metabolites.

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Abbreviations

2, 4-D	2, 4-dichlorophenoxy acetic acid
2-iP	2-isopentenyladenine
ABA	Abscisic acid
B5	Gamborg's medium
BA	Benzyladenine
DMSO	Dimethylsulfoxide
DW	Dry weight
FW	Fresh weight
GA	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LS	Linsmaier and Skoog medium
MS	Murashige and Skoog medium
PUFAs	Polyunsaturated fatty acids
SH	Schenk and Hildebrandt medium

20.1 Introduction

Secondary metabolites are a diverse group of organic compounds that are generally produced by plants to interact with biotic environment and for the establishment of defense mechanism [1]. Most secondary metabolites such as terpenes, phenolics and alkaloids which are classified based on their biosynthetic origin and chemical structures, show different biological activities and many of them have been used as pharmaceuticals, agrochemicals, flavours, fragrances, colours and food additives [2]. Secondary metabolite production is traditionally produced through field cultivation of plants, but this conventional approach suffers various disadvantages such as low yields, instability of their contents due to geographical, seasonal and environmental variations, occupation of large amount of lands, and heavy use of labors. Therefore, plant cell and organ cultures have emerged as an attractive alternative to whole plant cultivation for production of secondary metabolites [2]. However, there are still drawbacks in the production of metabolites through cell and organ cultures due to the instability of cell lines, low yields, slow growth and scale-up problems [3]. An important requirement in secondary metabolite synthesis is to understand the metabolic pathways and the enzymology of product formation as proposed by Dornenburg and Knorr [4], but unfortunately the knowledge of plant metabolic pathways is still

Table 20.1 Strategies to enhance the production of secondary metabolites in plant cell and organ cultures

Stage 1 – Biomass accumulation
1. Selection of efficient cell lines or clones
2. Medium optimization
(a) Selection of suitable medium and salt strength
(b) Carbohydrate source and concentration
(c) Nitrate levels
(d) Phosphate levels
(e) Growth regulator levels
3. Inoculum size
4. Optimization of the cultural environment
(a) Temperature
(b) Illumination
(c) Quality of light or combination of lights
(d) Medium pH
(e) Aeration and agitation
State 2 – Accumulation of bioactive compounds
5. Elicitation
6. Nutrient feeding
7. Precursor feeding
8. Permeabilization
9. Immobilization
10. Two phase system
11. Biotransformation
12. Organ cultures
13. Large-scale cultures

very limited. In spite of all these odds, cell and organ cultures have enormous potentialities for the production of industrially useful secondary compounds. Various strategies like strain improvement, optimization of medium and culture environments, elicitation for the accumulation of secondary compounds, nutrient and precursor feeding, permeabilization, immobilization and biotransformation methods have been developed over the years for high biomass accumulation and efficient synthesis of secondary compounds (Table 20.1). Biomass accumulation and biosynthesis of metabolites through cell and organ cultures may be looked at as a two step process in many cases: (1) involvement of cultured cells and organs in growth, multiplication and accumulation of biomass at the initial stage, and (2) biosynthesis of metabolites from the accumulated cells in the later stage. In the earlier reports, accumulation of biomass and production of secondary metabolite events were dealt simultaneously, however, it is possible to achieve both higher biomass accumulation and enhanced metabolite production by following a two step process, i.e. focusing on the accumulation of biomass strategy in the first step, and applying strategies for metabolite biosynthesis stimulation in the second step of the cultivation. The experimental strategies for the production of secondary metabolites by plant cell and organ cultures with suitable examples are summarized in this article.

20.2 Selection of Cell Lines and Clones

Initiation of cell and organ cultures begins with the choice of a parent plant with high content of the desired product for callus or organ induction to obtain high-yielding cell/organ lines. Secondary metabolite accumulation in plants is specific to its genotype. For example, the concentration of bacoside A (a triterpenoid saponin) varies among different genotypes ranging from 3.53 to 18.36 mg g^{-1} DW (Table 20.2) [5]. Similarly, the amount of camptothecin (a quinoline alkaloid) varies among the different species (*Camptotheca* spp., *Ervatamia* spp., *Ophiorrhiza* spp., *Nothapodytes* spp.) and even in different organs of the plant (0.03–0.4 % DW) [6]. Bacoside A is a nootropic and camptothecin is an anticancerous drug. A choice for suitable genotype and a suitable organ is essential for callus or organ induction to raise the cell or organ cultures for the production of these compounds.

Isolation and selection of cell and organ lines for growth (i.e. for higher biomass accumulation) as well as for accumulation of metabolites are most important. Earlier, selection of cell lines was carried out by visual screening if the product of interest would be a pigment. In *Euphorbia milli* and *Daucus carota*, enhanced anthocyanin production by clonal selection and visual screening has been reported [7, 8].

Table 20.2 Bacoside A concentration in *Bacopa monnieri* from different locations of Karnataka, India

Sl. No.	Accession no.	Location	Concentration of bacoside (mg g^{-1} DW)
1.	Bm1	Bangalore	6.55 ± 0.52
2.	Bm2	Belgaum	18.36 ± 1.65
3.	Bm3	Belgaum	5.19 ± 0.34
4.	Bm4	Belgaum	3.59 ± 0.41
5.	Bm5	Belgaum	7.81 ± 0.55
6.	Bm6	Belgaum	5.46 ± 0.42
7.	Bm7	Dharwad	3.53 ± 0.49
8.	Bm8	Dharwad	6.43 ± 0.36
9.	Bm9	Dharwad	5.70 ± 0.24
10.	Bm10	Gadag	6.83 ± 0.42
11.	Bm11	Haveri	10.56 ± 0.82
12.	Bm12	Haveri	6.90 ± 0.43
13.	Bm13	Haveri	6.67 ± 0.38
14.	Bm14	Kolar	8.52 ± 0.62
15.	Bm15	Mysore	7.24 ± 0.47
16.	Bm16	Shimoga	5.53 ± 0.26
17.	Bm17	Shimoga	7.93 ± 0.35
18.	Bm18	Shimoga	9.42 ± 0.77
19.	Bm19	Uttara Kannada	6.63 ± 0.34
20.	Bm20	Uttara Kannada	10.82 ± 0.86
21.	Bm21	Uttara Kannada	5.04 ± 0.31
22.	Bm22	Uttara Kannada	4.84 ± 0.18

However, selection made by analysis of growth of cell lines or root clones (adventitious or hairy roots) in suspension cultures followed by quantification of the desired product is considered to be superior to visual selection techniques. The growth kinetic analysis method is also followed in some cases. In *Orthosiphon stamineus* two cell lines were selected and identified which produced higher amount rosmarinic acid through cell suspension culture [9]. Quantification of metabolites by high pressure liquid chromatography and radioimmuno-assay are also followed for screening high yielding cell lines [10, 11].

20.3 Medium Optimization

A number of chemical and physical factors influence biomass accumulation and synthesis of secondary metabolites in plant cell and organ cultures. Medium composition is a basic and critical factor affecting the cell physiology and metabolism. Some of the key factors are choice of culture medium, suitable salt strength of the medium, sugar levels, nitrate levels, phosphate levels and growth regulator levels in the medium [2, 4, 12, 13].

20.3.1 Influence of Nutrient Medium and Salt Strength

Various types of media formulations were tested and utilized earlier for the establishment of cell and organ suspension cultures for the production of secondary metabolites. Murashige and Skoog (MS) [14], Gamborg's (B5) [15], Schenk and Hildebrandt (SH) [16], Linsmaier and Skoog (LS) [17] media are widely used. The B5 medium of Gamborg et al. [15] was initially used for callus and suspension cultures. This medium differs from MS medium in having much lower amounts of nitrates in the form ammonia hence, suitable for certain cell cultures. The appropriate concentration of medium constituents (salt strength) is crucial for the growth of isolated cells and organs. In ginseng adventitious root cultures, maximum biomass and growth rate were obtained in 0.75 strength MS medium and ginsenoside content and yield were higher in 0.5 salt strength MS medium [18]. The full strength MS medium was suitable for both biomass and gymnemic acid accumulation (Fig. 20.1) in cell suspension cultures of *Gymnema sylvestre* [19]. Among the 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 strength MS medium tested, full strength (1.0) medium was found better for biomass accumulation and withanolide A production in *Withania somnifera* cell suspension cultures [20]. Interestingly, some medium salts like calcium chloride and sodium chloride could be working as signal inducers to stimulate secondary metabolism. The inducing effects of calcium ion and sodium ion on the intracellular calcium signaling pathway were well demonstrated in higher plant and mushroom cell cultures for production of bioactive secondary metabolites like ginseng saponin and ganoderic acid [21, 22].

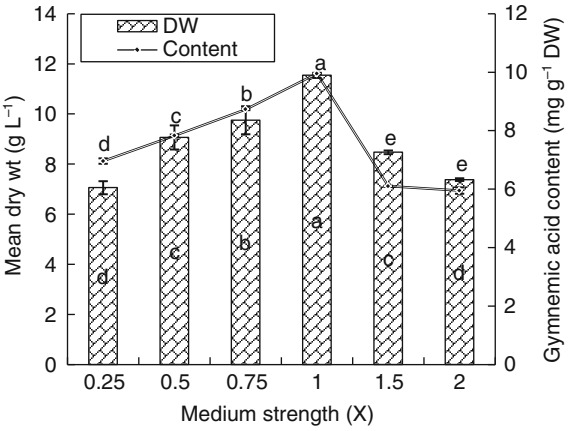

Fig. 20.1 Effect of medium strengths on biomass accumulation and gymnemic acid production in cell suspension cultures of *Gymnema sylvestre*. Five hundred mg of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} KN and 30 g L^{-1} sucrose for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

20.3.2 Influence of Carbon Source and Concentration

Plant cell cultures are usually grown heterotrophically using simple sugars such as glucose, fructose, maltose, sucrose and their combinations as an energy and carbon source. Among the various sugars tested, sucrose was found to be an ideal carbohydrate source for the biomass accumulation (11.56 g L^{-1} DW) and the highest production of gymnemic acid content (9.95 mg g^{-1} DW) (Table 20.3) [19]. Wang and Weathers [23] tested the effect of sugars on production of artemisinin in hairy root cultures of *Artemisia annua* and found a maximum production of artemisinin when hairy roots were grown in the medium supplemented with glucose, whereas the level of artemisinin produced in the medium supplemented with fructose was twice that in the medium supplemented with sucrose. Similarly, concentration of carbohydrate supplemented to the medium greatly affects the biomass and metabolite production. For example, of the various levels of sucrose (1–8 % w/v) tested in *Gymnema sylvestre* cell cultures, 3 % sucrose in the medium favoured the accumulation of biomass (Fig. 20.2), whereas the highest amount of gymnemic acid (10.1 mg g^{-1} DW) was accumulated in the medium supplemented with 4 % sucrose. In *Ginkgo biloba* cell cultures, 3 % sucrose was good for biomass accumulation whereas higher concentration of 5 and 7 % sucrose favoured the production of ginkgolides and bilobalides [24]. In *Bacopa monnieri* shoot cultures, 2 % sucrose was found optimal in the tested range (0–6 %, w/v) for biomass accumulation and sucrose-free medium accumulated maximum amount of bacoside-A [25]. The initial sucrose concentration (i.e. 20, 30, 40 and 60 g L^{-1}) had a significant effect on the production of ginseng saponin in suspension

Table 20.3 Effect of different carbohydrate sources on biomass accumulation and gymnemic acid production in *Gymnema sylvestre* cell suspension culture

Carbohydrate source (3 %)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Gymnemic acid content (mg g^{-1} dry weight)
Sucrose	125.67 a	11.56 a	10.16	9.95 a
Glucose	118.00 a	10.66 a	9.69	8.56 d
Fructose	100.91 b	9.23 b	8.39	6.58 f
Maltose	86.50 c	7.68 c	6.98	6.99 e
Glucose + fructose (1:1)	100.90 b	9.07 b	8.24	8.72 c
Fructose + sucrose (1:1)	94.25 c	8.36 c	7.60	9.26 b
Sucrose + glucose (1:1)	117.22 a	10.66 a	9.69	9.24 b

Cultures were grown in 250 mL conical flaks containing 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2, 4-D + 0.1 mg L^{-1} KN for 4 weeks. Mean values with common letter within each column are not significantly different at $P \leq 0.05$ according Duncan's multiple range test

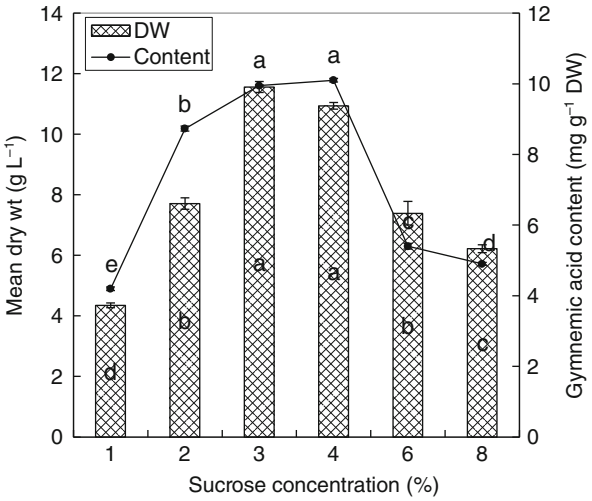

Fig. 20.2 Effect of different sucrose concentrations on the biomass accumulation and gymnemic acid production in cell suspension cultures of *G. sylvestre.* Five hundred mg of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

cultures of *Panax notoginseng* [26]. A high sugar level was favorable to the synthesis of ginseng saponin, may be due to the high osmotic pressure which was caused by high sugar concentration [27]. But, the cell growth was reduced at an initial sucrose concentration of 60 g L^{-1}, the maximum production of crude ginseng saponins (0.86 g L^{-1}) was achieved at an initial sucrose concentration of 40 g L^{-1} [26]. The osmotic stress created by sucrose alone or with other osmotic agents was found to regulate anthocyanin production in *Vitis vinifera* cell

suspension cultures [28]. A dual role of sucrose as a carbon source and an osmotic agent was observed in *Solanum melongena* [29]. Recently, sugars have been recognized as signaling molecules that affect growth, development and metabolism of cultured cells [23]. Therefore, the selection of a suitable carbohydrate source at appropriate concentration is a key criterion for secondary metabolite production in cell and organ cultures.

20.3.3 Influence of Nitrogen Source

The growth and metabolite accumulation in cell and organ suspension cultures was found to be influenced by a suitable nitrogen source and its concentration. The plant tissue culture media such as MS, LS, SH, and B5 contain both nitrate and ammonium as source of nitrogen. However, nitrogen present in the ammonium/nitrate and overall levels of total nitrogen have markedly affected both biomass accumulation and production of secondary plant products. In the shoot cultures of *Bacopa monnieri*, the effect of macro elements was tested by varying the levels of NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4 in the MS medium each at 0.05, 1.0, 1.5 and 2.0 strengths and optimum number of shoots (99.33 shoots explant^{-1}), biomass (0.150 g DW) and the highest production of bacoside A (17.9 mg g^{-1} DW) were obtained with 2× strength NH_4NO_3 [30]. The effect of nitrogen supplements like $NH4^+$/$NO3^-$: 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 (mM mM^{-1}) when tested they found that shoot biomass and bacoside A content were optimum when the $NO3^-$ concentration was higher than that of $NH4^+$ (ratio of 14.38/37.60 mM; Fig. 20.3). In another report, reduced level of $NH4^+$ and increased levels of $NO3^-$ promoted the production of gymnemic acid and withanolide A [31, 32, 33]. Reduced levels of total nitrogen improved the production of capsaicin in *Capcicum frutescens*, and anthraquinones in *Morinda citrifolia* [34, 35]. However, complete elimination of nitrate in cultures of *Chrysanthemum cinerariaefolium* induced a twofold increase in pyrethrin accumulation in the second phase of culture [36]. The effects of the nitrate to ammonium ratio on the cell growth, the production of ginseng saponin and polysaccharide as well as consumption of major nutrients by suspension cultures of *Panax notoginseng* cells were investigated at total nitrogen of 60 mM in a 250-mL Erlenmeyer flask [37]. The biosynthesis of saponin was more susceptible to the ratio of NO_3^-/NH_4^+ than that of polysaccharides. Ammonium was unfavorable for saponin formation. The relationship between initial nitrate concentration (including both intracellular and medium nitrate) and specific cell growth rate based on active biomass could be described by Monod equation. The maximum production of crude saponin and polysaccharide was 0.85 and 1.59 g L^{-1}, respectively, with initial nitrate concentration of 60 mM.

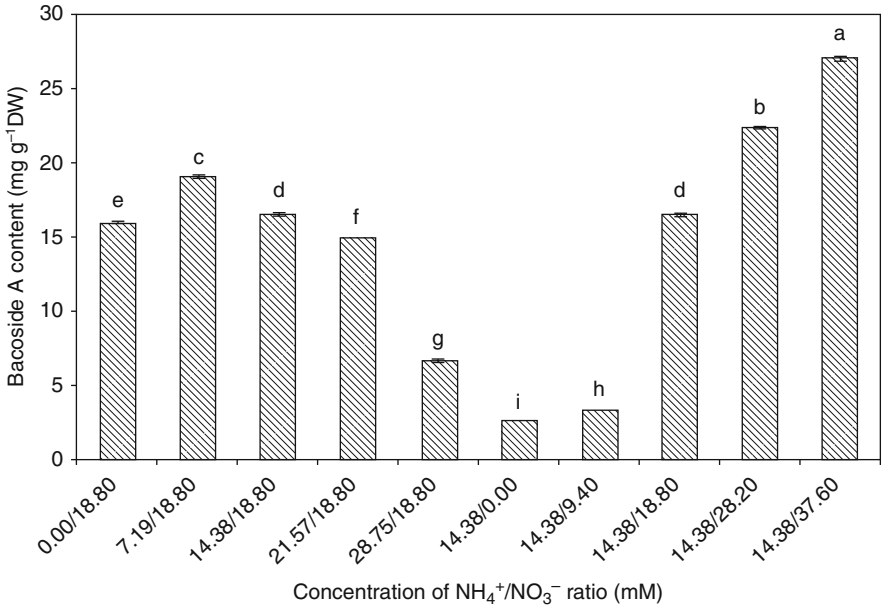

Fig. 20.3 Bacoside A content in *Bacopa monnieri* adventitious shoot culture after 8 weeks of cultivation as affected by different ratio of NH_4^+/NO_3^- in the MS medium. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

20.3.4 Influence of Phosphate Levels

The phosphate concentration in the medium shows an excitatory effect on the production of secondary metabolites in plant cell and organ cultures. An increased phosphate level has been shown to stimulate synthesis of digitoxin in *Digitalis purpurea* [38]. Liu and Zhong [39] have reported that the highest saponin production at an initial phosphate concentration of 1.04 mM in *Panax ginseng* and 1.25 mM *Panax quinquefolium* respectively. Twice the phosphate levels of standard MS medium (1.25 mM) has proved better for the production of rosamarinic acid from *Lavandula vera* suspension cultures [31], gymnemic acid production from *Gymnema sylvestre* cell cultures [40] and solamargine production by *Solanum paludosum* multiple shoot cultures [41]. On the other hand, there are a number of reports showing that phosphate limitation could improve the production of metabolites, caffeine content in cell suspension cultures of *Coffea arabica* increased under phosphate limitations [42]. In grape cell suspensions, Dedaldechamp et al. [43] have reported enhancement of anthocyanin synthesis in response to phosphate deprivation.

20.3.5 Influence of Growth Regulator Levels

Growth regulators affect the growth and metabolite accumulation significantly because cell, adventitious root or shoot cultures generally need exogenous supply of growth regulators for growth, proliferation of biomass and metabolite accumulation. Whereas, hairy root cultures are genetically transformed roots which are produced by using *Agrobacterium rhizogenes* and they have the ability to grow without the addition of plant growth regulators [44]. However, a few recent reports showed that exogenous application of growth regulators also influence the growth and metabolite accumulation in hairy root cultures [45, 46]. In general, type of plant growth regulator and its concentration are crucial factors in cell and organ growth, proliferation and metabolite accumulation [47]. The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alter dramatically both the growth and the product formation in cultured cells [48]. Among auxins, indole acetic acid (IAA) and naphthalene acetic acid (NAA) have shown triggering effect on the production of anthocyanins in suspension cultures of populus and carrot, nicotine in tobacco, and anthraquinones in noni [34, 49, 50]. 2, 4-Dichlorophenoxyacetic acid (2, 4-D) has also shown a stimulatory effect on the accumulation of carotenoids in carrot [51] and anthocyanin in oxalis [52]. In suspension cultures of *Panax quinquefolium* strain Q91625, the highest content of crude ginsenoside saponins, i.e. 10.9 % by dry weight, was reached under a combination of the growth regulators of 2.5 mg L^{-1} indole-3-butyric acid (IBA) and 0.1 mg L^{-1} kinetin without addition of 2,4-D [53]. Among cytokinins, benzyladenine (BA) addition has improved the production of saponins in ginseng and addition of kinetin stimulated the production of anthocyanin in slender golden weed, but inhibited the production of anthocyanins in populus [49, 51]. 2-Isopentenyladenine (2-iP) inhibited root growth, however, stimulated artemisinin production in *Artemisia annua* [46].

The effect of gibberellins (GA) was specific to species and culture time. For example, Vanhala et al. [45] observed that addition of GA_3 decreased the accumulation of hyoscyamine in henbane. In contrast, GA_3 stimulated production of artemisinin in *Artemisia annua* and coumarin content in *Cichorium intybus* [54, 55]. Ethylene stimulated artemisinin production in plantlet cultures of *A. annua* [56] and it enhanced the growth of hairy roots of *Hyoscyamus muticus* [57]. A little is known about the effects of exogenous abscisic acid (ABA) on cell and organ cultures. Usually ABA inhibits growth and accumulation of secondary metabolites. ABA inhibited hyoscyamine accumulation in hairy root culture of *H. muticus* [45] with no adverse effect on biomass. In *Lotus corniculatus*, ABA application stimulated growth, but inhibited the accumulation of tannin [58].

20.4 The Influence of Inoculum Size/Density

Inoculum size/density is an important factor for plant cell and organ suspension cultures, which can influence the growth, biomass accumulation and metabolite formation [59, 60]. There is a critical minimum inoculum size below which cell

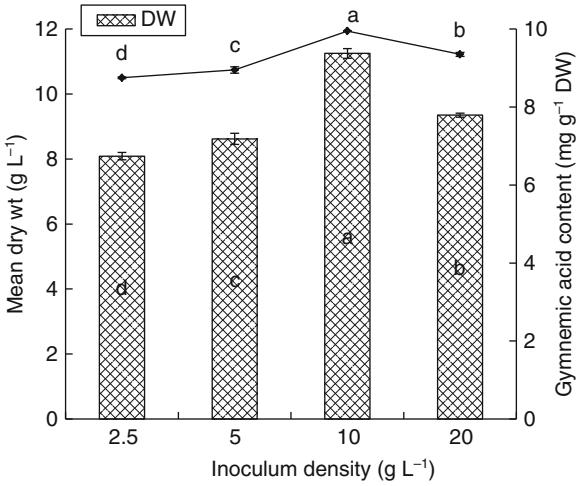

Fig. 20.4 Effect of inoculum density on the biomass and gymnemic acid accumulation in cell suspension cultures of *G. sylvestre*. Five hundred mg of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

growth will normally fail, while a suitable level of inoculum density could interestingly promote not only the cell growth but also the secondary metabolite biosynthesis. There are many reports on the influence of inoculum size/density of the cultured cells on biomass and metabolite accumulation [61–64]. In suspension cultures of *Perilla frutescens*, maximum cell density of 38.3 g DW L^{-1} was obtained at an elevated inoculum size of 50 g wet cells L^{-1} and anthocyanin production was enhanced 23-fold [65]. In cell suspension cultures of *Gymnema sylvestre*, the various quantities of inoculums (2.5, 5.0, 10.0 and 20.0 g L^{-1}) were tested, optimum density of biomass (11.25 g L^{-1}) as well as gymnemic acid (9.95 mg g^{-1} DW) was achieved with 10.0 g L^{-1} inoculum (Fig. 20.4). A higher (20.0 g L^{-1}) and lower (2.5 g L^{-1}) inoculum was not suitable for biomass and gymnemic acid accumulation. Another effect of inoculum size/density was at the induction of enzymes involved in the general phenylpropanoid metabolism when cells were transferred to a fresh medium. This is called 'transfer effect' or 'dilution effect'. Hahlbrock and Wellmann [66] have reported that the phenylalanine ammonia-lyase induced by transfer to fresh medium, decreased with increased inoculum size signifying that inoculum density may affect secondary metabolism. Morphology of the roots is another factor which influences biomass growth and synthesis of secondary compounds [67, 68] in the root suspension cultures. The adventitious root inoculum which was chopped (1–3 or 4–6 mm) or un-chopped, were responsible for lower yield of dry weight as well as ginsenosides. The root inoculum chopped to 7–10 mm was responsible for higher yield of 10 g L^{-1} DW and they also possessed highest content of ginsenosides of 5.5 mg g^{-1} DW [68].

20.5 Optimization of Culture Environment

Conditions of culture environment such as light, temperature, medium pH, and gases have been examined for their effects on biomass and secondary metabolite accumulation in cell and organ cultures.

20.5.1 Influence of Temperature

Since the early development of plant biotechnology, temperature effect has been investigated in cell and organ cultures and a temperature range of 17–25 °C is normally used for the maintenance of cultured cells and organs. However, each plant species may show better growth and metabolism under different temperature regimes. Morris [69] studied *Catharanthus roseus* cell line C87 and found maximum growth rate at 35 °C, maximum dry weight yield (0.47 g g^{-1}) was observed at 25 °C. Scragg et al. [70] investigated *Catharanthus roseus* cell line ID1 at 20, 25 and 30 °C but maximum biomass yield of 0.65 g g^{-1} at 25 °C. Courtois and Guern [71] found an optimum temperature of 16 °C for production of ajmalicine. Morris [69] reported that an optimum temperature of 25 °C for serpentine production and 20 °C for ajmalicine production. Toivonen et al. [72] estimated an optimum temperature of 25 °C for production of alkaloid from cell suspension cultures of *Catharanthus roseus*. Shohael et al. [73] studied the effect of low temperature (12 and 16 °C) and higher (30 °C) temperature, and reported that low and high temperatures cause significant decrease in biomass and reduction of phenolics and flavonoids, while low temperatures boost the accumulation of eleutheroside E in somatic embryos of *Eleutherococcus senticosus* and they correlated the increased accumulation of eleutheroside E for the oxidative stress. Yu et al. [74] studied the growth of hairy roots of ginseng under differential temperatures such as 13/20, 20/13, 25/25, and 30 °C/25 °C for 16/8 day and night cycles; got highest hairy root biomass with the cultures incubated at 20 °C/13 °C (Table 20.4). However, total ginsenosides was optimum (10.5 mg g^{-1} DW; Table 20.4) with the cultures incubated at 25 °C/25 °C and ginsenoside production was also highest (133.4 mg L^{-1}) at this temperature.

Table 20.4 Effect of incubation temperature (with 16 h/8 h/night cycles) on growth and ginsenoside production of ginseng hairy roots cultivated in bioreactors for 4 weeks

Growth	Biomass		Growth ratio	Ginsenoside (mg g^{-1} DW)	Ginsenoside (mg L^{-1})
	FW (g)	DW (g)			
13/20	431 ± 1.0	28 ± 1.0	19.7	4.5 ± 0.1	31.5 ± 1.5
20/13	892 ± 0.9	65 ± 0.8	45.8	8.2 ± 0.1	133.9 ± 0.9
25/25	889 ± 0.6	51 ± 0.7	35.9	10.5 ± 0.1	133.4 ± 1.2
30/25	764 ± 0.8	64 ± 0.9	45.1	6.4 ± 0.1	71.6 ± 0.5

Values within each column represent the mean of three replicates ± S.E.

20.5.2 *Influence of Light Intensity and Quality*

Light may be used as an energy source or just as an elicitor which affects the growth and accumulation of secondary metabolites in cultured cells and organs. Zhong et al. [75] demonstrated the effects of light quality, intensity, and irradiation period on the cell growth and anthocyanin pigment production by suspended culture of *Perilla frutescens*, and finally they optimized and successfully scaled-up the cell culture process from shake flasks to bioreactors based on the key factor of light irradiation. Chan et al. [76] also investigated the effects of different light intensity and irradiance (continuous radiance and continuous darkness) on cell biomass yield and anthocyanin production in cultures of *Melastoma malabathricum*. Moderate light intensity (300–600 l×) induced higher accumulation of anthocyanins, the cultures exposed to continuous darkness for 10-days showed the lowest pigment content, while the cultures exposed to continuous irradiance for 10-days showed the highest pigment content. The stimulatory effect of light on the formation of secondary compounds has been reported including flavonoids in *Petroselinum hortense* [77], anthocyanins in *Centaurea cyanus* [78], betalains in red beet [79], artemisinin in *Artemisia annua* [80]. On the contrary, light has an inhibitory effect on the accumulation of secondary metabolites such as nicotine and shikonin in *Lithospermum erythrorhizon* [81], monoterpenes in *Citrus limon* [82]. In some species, such as *Fragaria ananassa* [83]; and sweet potato [84], cell cultures have been reported to produce anthocyanin in the dark. Yu et al. [74] have studied the effect of fluorescent light, metal halide light, blue light, red light and blue plus red light on biomass growth and synthesis of ginsenosides in ginseng hairy root cultures and reported that hairy root growth was stimulated by red light than dark (Table 20.5). Fluorescent irradiation enhanced the accumulation of ginsenosides (5.3 mg g^{-1} DW). They also noticed differential accumulation of Rb and Rg group of ginsenosides in dark grown and light grown cultures, Rb group ginsenosides were highest in the cultures grown in dark (4.5 mg g^{-1} DW; Table 20.5) and Rg group of ginsenosides were optimal in the cultures grown in light (5.3 mg g^{-1} DW). These results suggest that manipulation of secondary metabolite accumulation is possible by manipulating light and dark regimes.

Table 20.5 Effect light quality on growth and ginsenoside production in ginseng hairy roots cultivated in bioreactors for 4 weeks

Light sources	Biomass		Growth ratio	Ginsenoside (mg g^{-1} DW)		Ginsenoside production (mg L^{-1})
	FW (g)	DW (g)		Rg	Rb	
Dark	270 ± 1.0	24 ± 0.6	11.4	2.8 ± 0.1	4.5 ± 0.2	27.8 ± 1.0
FL	226 ± 0.8	21 ± 0.6	10.1	5.3 ± 0.1	3.7 ± 0.1	30.2 ± 0.9
MH	193 ± 1.1	19 ± 0.3	8.9	3.5 ± 0.4	3.4 ± 0.3	23.3 ± 0.2
B	236 ± 0.2	24 ± 0.9	11.3	3.8 ± 0.4	3.9 ± 0.5	26.6 ± 0.4
R	284 ± 0.9	25 ± 1.0	11.6	3.1 ± 0.8	4.1 ± 0.7	20.9 ± 0.4
B + R	183 ± 0.9	21 ± 0.9	10.1	3.4 ± 0.1	2.9 ± 0.2	24.2 ± 0.7

Values within each column represent the mean of three replicates ± S.E.
FL fluorescent light, *MH* metal halide light, *B* blue light, *R* red light, *B + R* blue plus red light

20.5.3 *Influence of Hydrogen Ion Concentration*

The medium pH is usually adjusted between 5 and 6 before autoclaving and extremes of pH are avoided. The concentration of hydrogen ions in the medium changes during the course of culture due to nutrient uptake or due to the accumulation of metabolites in cultures. For example, decrease of medium pH due to ammonium assimilation and increase due to nitrate uptake was reported by McDonold and Jackman [85]. In *Withania somnifera* hairy root cultures, initial pH of the medium which was set at 5.8 was favourable for the accumulation of biomass (12. 1 g L^{-1} DW) and medium pH of 6.0 favoured the accumulation of withanolide A in the roots (13.84 mg g^{-1} DW; Fig. 20.5) [86]. In hairy root cultures of *Tagetes patula,* medium pH of 5.7 was suitable for growth and accumulation of thiophene [87]. In hairy root cultures of *Panax ginseng*, the medium pH set at 6.0 and 6.5 favoured both biomass accumulation and ginsenoside production [88]. The strategy of alteration of medium pH which results in the release of secondary products into the culture medium by changing the membrane permeability of the cells was reported in many culture systems [89, 90]. For example, betalains normally accumulate in roots of *Beta vulgaris*, but are released into the medium at pH 5.5 [90]. Up to 50 % of the total pigment was released at the time of exposure and roots continued to grow and accumulate betalains at later stage. When the roots were exposed to pH 2 for 20–30 min, they failed to grow, suggesting that low pH causes lysis of mature-pigment cells. A short exposure (10 min) to pH 2 followed by return to standard growth medium (pH 5.5) was beneficial for continuous release of pigments in the medium.

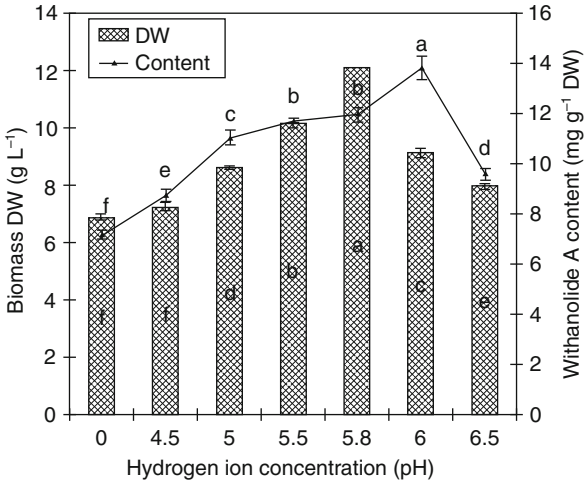

Fig. 20.5 *Withania somnifera* hairy root cultures: effect of pH on biomass accumulation and withanolide A production. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer flasks containing 50 ml of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values of three replicates. Means values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

20.5.4 *Influence of Agitation and Aeration*

Agitation is one of the important criteria which should be controlled in flask-scale to large-scale bioreactor cultures. The mixing of cultures promotes better growth by enhancing the mass transfer and uptake of nutrients from liquid and gaseous phases by cells/organs and the dispersion of air bubbles for effective oxygenation. Although plant cells have higher tensile strength in comparison to microbial cells, their shear sensitivity to hydrodynamic stresses restricts the use of high agitation speed for efficient mixing. The high shear rate and shear time that accompanies good mixing reduce the mean aggregate size, but also have an adverse effect on cell viability. Plant cells are therefore, often grown in stirred tank bioreactors at very low agitation speeds. Shifting from cell cultures to organ cultures such as adventitious or hairy root, shoot and embryo cultures for the production of secondary metabolites may be advantageous to overcome rheological problems [91, 92]. Many bioprocess techniques have been worked out by chemical engineers to overcome shear sensitivity, oxygen supply and mixing problems for the cultivation of plant cells in bioreactors [93–97].

Aeration is another important factor which should be controlled in bioreactor cultures for culture process optimization [93, 96, 97]. Aeration of plant cell cultures fulfills three main functions: maintenance of aerobic conditions, desorption of volatile products and removal of metabolic heat by mixing and air flow [97]. Oxygen requirement of plant cells is comparatively lower than that of microbial cells due to their low respiratory rates. However, oxygen supply has been shown significantly affecting the secondary metabolite production in cell cultures [93, 98, 99]. The effects of oxygen supply within the range of 20.8 %, 30, 40 and 50 % was studied by Thanh et al. [100] with ginseng cell cultures and a 40 % oxygen supply was found to be beneficial for the production of both cell biomass and spawning yield respectively (Fig. 20.6). In some cases, high oxygen concentration was even

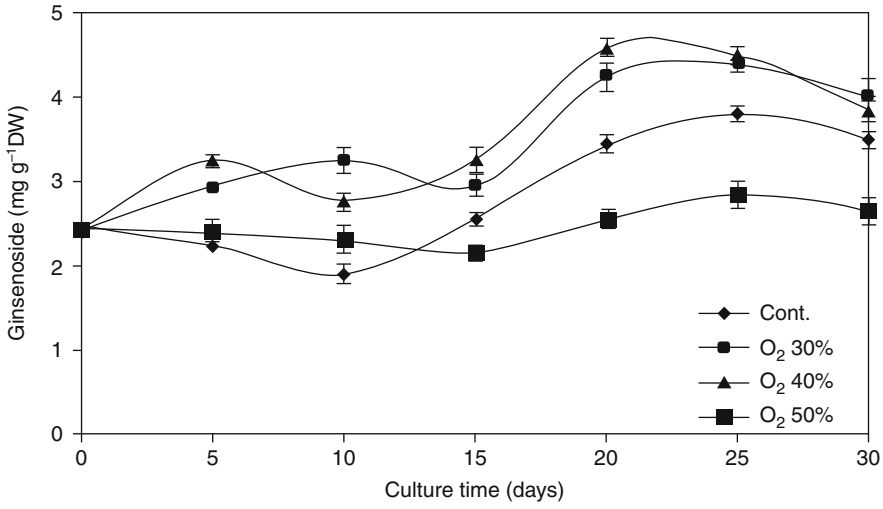

Fig. 20.6 Kinetics of ginseng saponin production by bioreactor-cultivated *Panax ginseng* cells

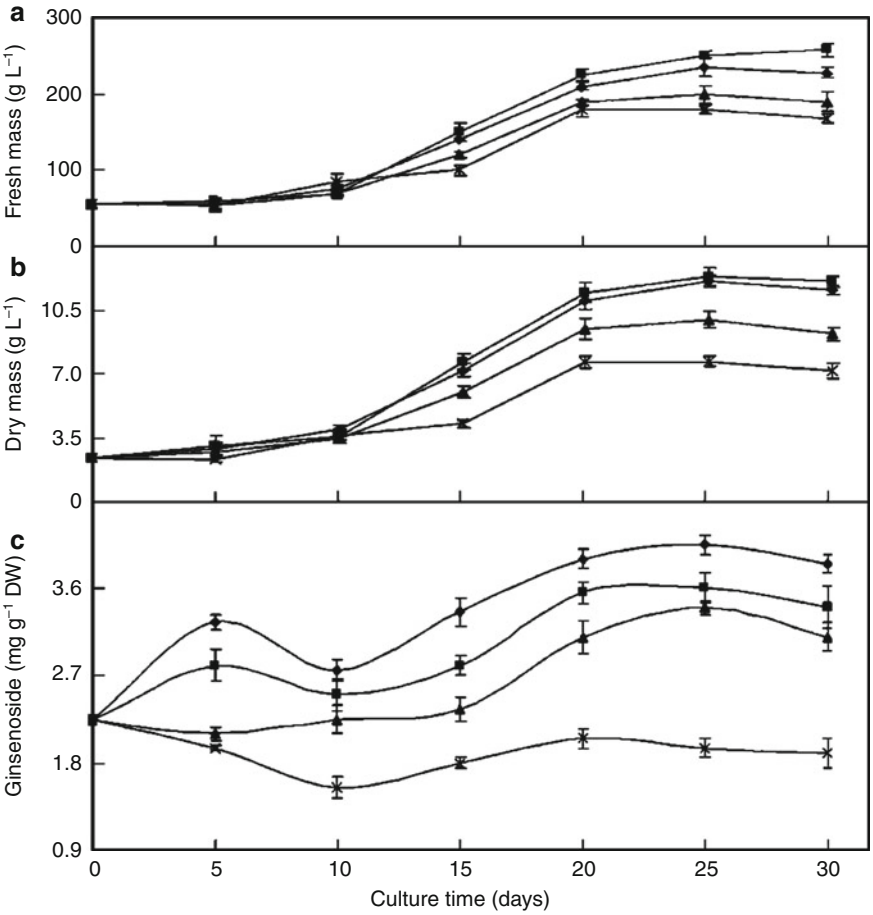

Fig. 20.7 Effect of CO_2 concentration on accumulation of cell fresh mass (**a**), dry mass (**b**) and production of saponins (**c**) of *Panax ginseng* cells cultivated in balloon type bubble bioreactors (*rhomboidal dots* – control, CO_2, *square* – 1 % CO_2, *triangle* – 2.5 % CO_2, *cross* – 5 % CO_2)

toxic to the metabolic activities of cells and may strip nutrients such as carbon dioxide from the culture broth [97]. Carbon dioxide is often considered as an essential nutrient in plant cell culture and has a positive effect on growth. The effects of carbon dioxide supply at 0.03, 1.0, 2.5 and 5.0 % in ginseng cell cultures was analyzed by Thanh et al. [101] and on improvement in biomass accumulation with 1 % carbon dioxide supply was observed. However, supplementation of carbon dioxide was not beneficial for saponin accumulation (Fig. 20.7). The beneficial effect of carbon dioxide on secondary metabolite production has been demonstrated in cell cultures of *Thalictrum minus* [102], *T. rugosum* [103], and *Stizoloibum hassjoo* [104].

20.6 Elicitation

Secondary metabolites are synthesized and accumulated in plant cells in response to a varied kind of stresses like biotic stresses such as a pathogen or insect attack and abiotic stresses like temperature, salinity, water stress, radiation stress, heavy metal and mineral stresses [105]. These varied stress conditions are generally designated as 'elicitors' [4] and elicitation has been widely used to enhance the production of secondary metabolites in plant cell and organ cultures [4, 105]. Elicitors of fungal, bacterial or yeast origin, viz. polysaccharides, glycoproteins, inactivated enzymes, purified crudlan, xanthan and chitosan salts and heavy metals are reported for the enhanced production of various secondary metabolites. Signaling molecules like methyl jasmonate and salicylic acid are also widely used for increased accumulation of secondary metabolites in cell and organ cultures [106–110]. Elicitor concentration, duration of exposure and age or stage of culture for elicitor treatment is also important for the successful production of secondary metabolites. Yu et al. [106] have studied the effect of jasmonic acid (0, 1.0, 2.0, 5.0 and 10.0 mg L^{-1}) on ginseng adventitious root cultures and increase in concentration of jasmonic acid resulted in a decrease in both fresh and dry biomass (Table 20.6). Whereas, ginsenoside content increased with higher concentrations up to 5.2-fold (Table 20.7). Decrease in biomass was tackled

Table 20.6 Effect of jasmonic acid on growth of ginseng adventitious roots after 5 weeks of flask culture

	Biomass		
Jasmonic acid (mg L^{-1})	Fresh weight (g)	Dry weight (g)	Growth ratio
0.0	16.15 ± 0.03	1.47 ± 0.07	4.08
1.0	12.48 ± 1.03	1.02 ± 0.07	2.83
2.0	8.52 ± 0.15	0.89 ± 0.01	2.47
5.0	6.33 ± 0.77	0.59 ± 0.07	1.64
10.0	4.67 ± 0.09	0.41 ± 0.01	1.13

Values within the columns represent the mean of three replicates ± S.E

Table 20.7 Effect of jasmonic acid on the biosynthesis of ginsenosides after 5 weeks of culture

Jasmonic acid (mg L^{-1})	Ginsenoside content (mg g^{-1} DW)			Rb/Rg	Ginsenoside production (mg L^{-1})
	Rb	Rg	Total		
0.0	7.49 ± 0.89 c	3.92 ± 0.34 c	11.42 ± 0.55 c	1.95 ± 0.39 c	167.58 ± 8.04 c
1.0	13.29 ± 0.49 d	2.83 ± 0.02 d	16.09 ± 0.46 d	4.68 ± 0.21 d	164.12 ± 4.69 c
2.0	24.29 ± 0.94 c	4.46 ± 0.25 b	28.69 ± 1.16 c	5.45 ± 0.16 c	255.39 ± 9.32 a
5.0	34.69 ± 0.89 b	4.15 ± 0.26 bc	38.82 ± 1.34 b	8.43 ± 0.71 b	229.04 ± 7.91 b
10.0	54.29 ± 1.04 a	5.53 ± 0.14 a	59.87 ± 0.90 a	9.83 ± 0.43 a	245.47 ± 3.69 a

Mean values of three replicates are represented with standard error

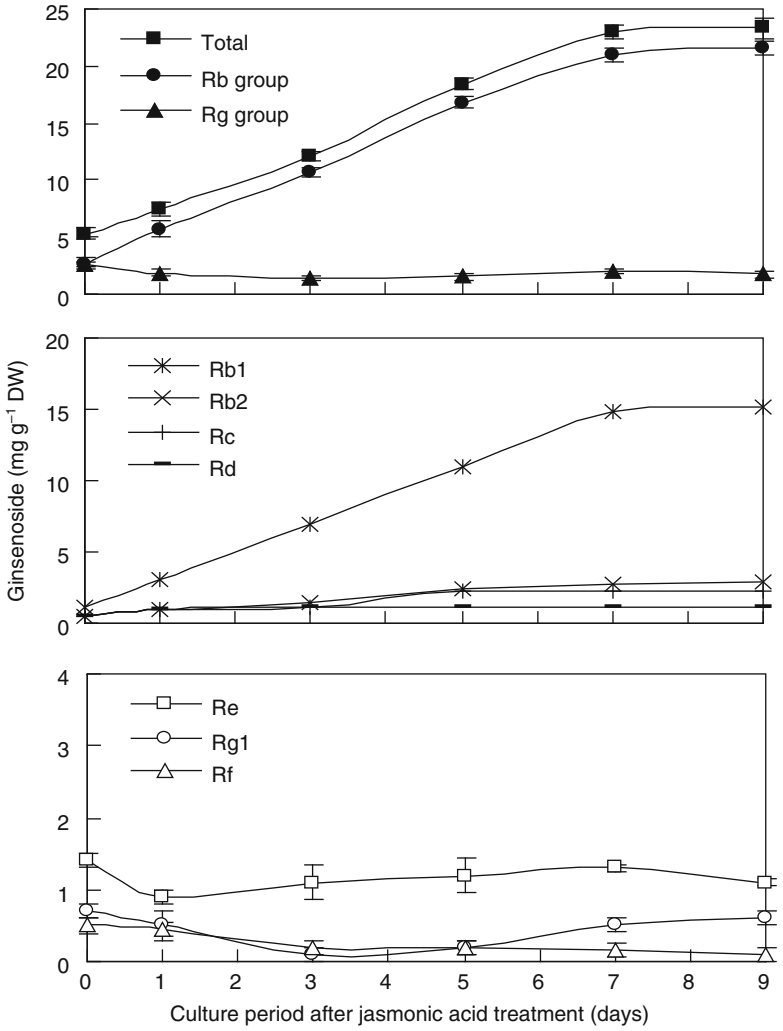

Fig. 20.8 Accumulation of total, Rb and Rg group ginsenosides during 9 days cultivation after jasmonic acid treatment (2 mg L^{-1}). The adventitious roots were grown for 25 days before jasmonic acid treatment

by following two step strategies i.e. by growing the adventitious roots in cultures for 25 days without elicitor and then by adding jasmonic acid (2 mg L^{-1}) and later there was increase in total ginsenosides and Rb group of ginsenosides by 5 and 5.6-fold respectively (Fig. 20.8). Hence, by following two step strategies it was possible to achieve both biomass growth and ginsenoside accumulation. Similarly, jasmonates have been used to elicit accumulation of paclitaxol in cell cultures of various *Taxus* species [111, 112], saikosaponins in root cultures of *Bupleurum falcatum* [113], eleutherosides in embryo cultures of *Eleutherococcus senticosus* [114].

Zhong and his co-workers have successfully developed a couple of new elicitors by chemical structure modification of traditional methyl jasmonate [115–117]. The novel chemically synthesized hydroxyl-containing jasmonates are more powerful

Table 20.8 The effect of linoleic and α-linolenic fatty acids on biomass production of *Panax ginseng* adventitious roots

Fatty acid concentration (μ mol L^{-1})	Biomass			Growth ratio
	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	% dry weight	
Control				
0.0	134.3 ± 0.3 c	9.9 ± 0.3 cd	7.4 ± 0.2 abc	18.2 ± 0.6 cd
Linoleic acid (18:2) ±				
1.0	128.5 ± 1.2 d	9.6 ± 0.1 de	7.4 ± 0.1 abc	18.1 ± 0.1 cde
2.5	125.5 ± 1.2 d	8.8 ± 0.3 de	7.0 ± 0.2 dc	16.6 ± 0.7 de
5.0	120.7 ± 0.3 ef	8.8 ± 0.1 de	7.3 ± 0.1 bc	16.6 ± 0.7 de
10.0	120.8 ± 0.6 ef	8.7 ± 0.1 de	7.2 ± 0.1 bc	16.6 ± 0.1 de
20.0	120.1 ± 2.0 f	8.6 ± 0.1 e	7.1 ± 0.1 c	16.2 ± 0.1 e
α-Linolenic fatty acid (C18:3)				
1.0	136.9 ± 0.3 c	11.2 ± 0.3 ab	8.2 ± 0.2 a	21.4 ± 0.6 ab
2.5	150.3 ± 1.6 a	11.9 ± 0.6 a	8.0 ± 0.5 ab	22.9 ± 1.1 a
5.0	145.2 ± 3.2 b	11.1 ± 0.9 ab	7.7 ± 0.5 abc	21.6 ± 1.9 ab
10.0	145.7 ± 0.3 b	11.6 ± 0.1 ab	7.9 ± 0.1 abc	22.1 ± 0.7 ab
20.0	138.9 ± 1.0 c	10.4 ± 0.1 c	7.5 ± 0.1 abc	19.9 ± 0.1 bc
Significance (ANOVA)				
Elicitor (E)	***	***	***	***
Elicitor concen. (EC)	**	**	**	**
E × EC	**	*	–	–

Adventitious roots were cultured in 5 l balloon-type bioreactors containing 4 L MS liquid medium supplemented with 5 mg L^{-1} IBA and 5 % sucrose. Filter sterilized elicitors were added to culture medium on day 40. Roots were harvested and assayed at day 47. Mean values of three replicates are with ANOVA, at $P \leq 0.05$

than methyl jasmonate in inducing taxoid biosynthesis [115], and a new strategy of repeated elicitation plus sucrose feeding greatly enhanced the taxoid production titer to nearly 1 g L^{-1} in cell cultivation of *Taxus chinensis* [116]. The dynamic responses of defense signals and biosynthetic gene transcription to the new elicitor addition were also elucidated in the plant cell cultures [117]. Recently, polyunsaturated fatty acids (PUFAs) are known to possess biological activities in tissue cultures. For instance, exogenous PUFAs increased accumulation of secondary metabolites in suspension cultures of *Lycopersicon esculentum*, *Tinospora cordifolia*, *Erythrina cristagalli* and *Eschscholzia californiaca* [118]. In addition, elicitation with α-linolenic acid enhanced the activity of lipoxynase, the key enzyme of oxilipin biosynthesis [119]. When linoleic and α-linolenic acid were used as elicitors at a concentration ranging from 0 to 20 μM L^{-1} in adventitious root cultures of *Panax ginseng*, it was found that the effect of linoleic and α-linolenic acid was concentration dependent. In the cultures, linoleic acid significantly reduced root biomass growth and α-linolenic acid promoted biomass growth (Table 20.8) [120]. The content of protopanaxadiol and protopanaxatriol ginsenosides was elevated with the addition of α-linolenic acid (Fig. 20.9). Similarly, in the cell cultures of *Agrostis tenuis*, *Rauvolfia serpentina* and *Nicotiana tabacum*, addition of α-linolenic acid induced accumulation of jasmonic acid and was accountable for biosynthesis of pentacyclic oxylipins [118].

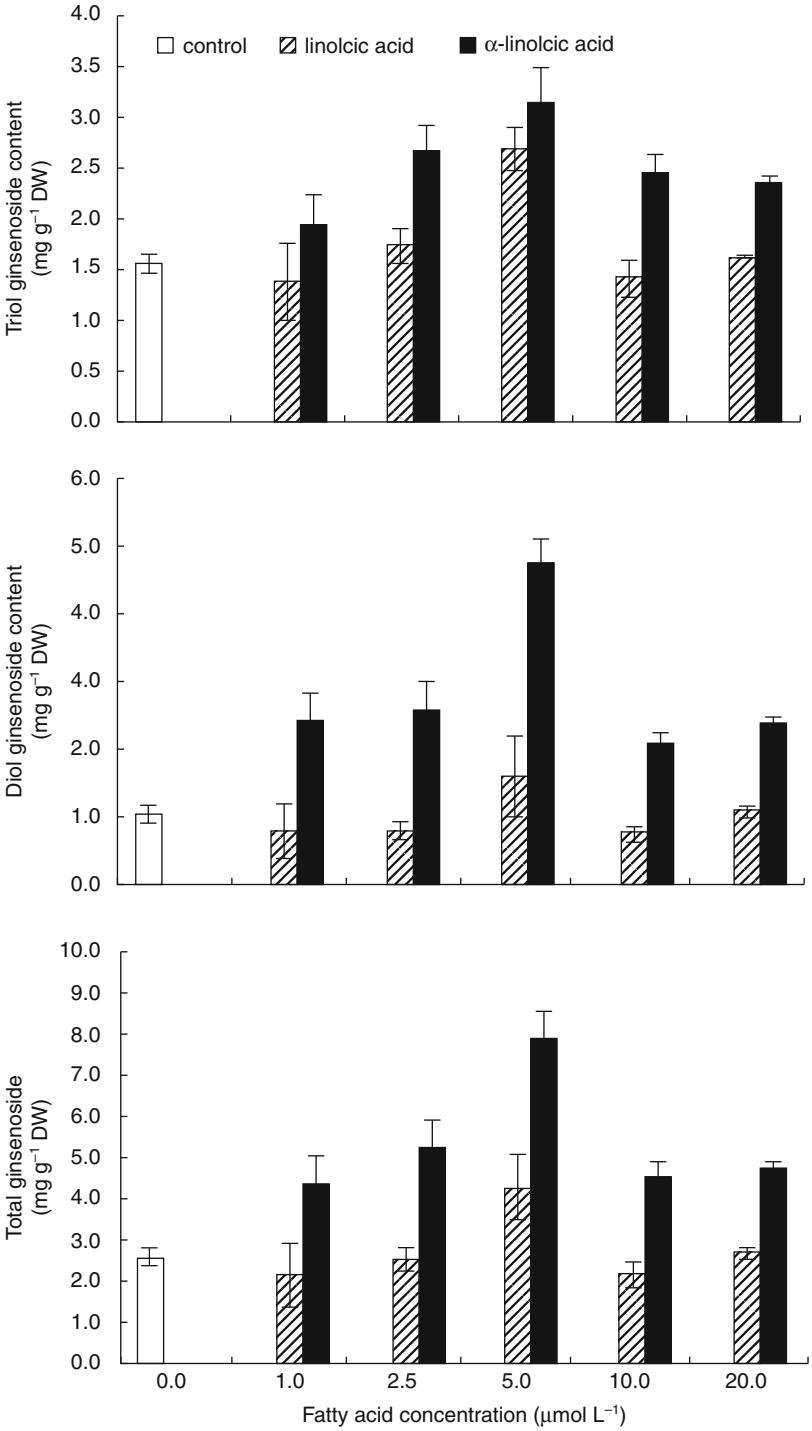

Fig. 20.9 Ginsenoside content of *Panax ginseng* adventitious roots as affected by elicitation with linoleic acid and α-linolenic acid fatty acids. All values are the means from three replicates with their standard errors

20.7 Nutrient Feeding

Medium or nutrient feeding strategy is one among the various approaches used to enhance the production of secondary metabolites after optimizing the basic chemical and physical parameters for the cultivation of cells/organs in large-scale [93, 121]. For instance, various nutrients of culture medium were exhausted by the end of 40 days of culture during ginseng adventitious root cultures (Fig. 20.10); with the objective of meeting the nutrient requirements of ginseng adventitious root cultures, and enhancing the biomass as well as ginsenosides production, Jeong et al. [121] replenished the cultures with 0.75 and 1.0 strength media after 10 and 20 days of cultivation. The cultures replenished with fresh medium (1.0-strength MS medium after 20 days of culture) showed a 27.45 % increase in dry biomass (28.66 g L^{-1} with replenishment treatment) and 8.25 % increase in ginsenoside content (4.93 gm g^{-1} DW; Table 20.9). The similar positive effect of media exchange strategy has been

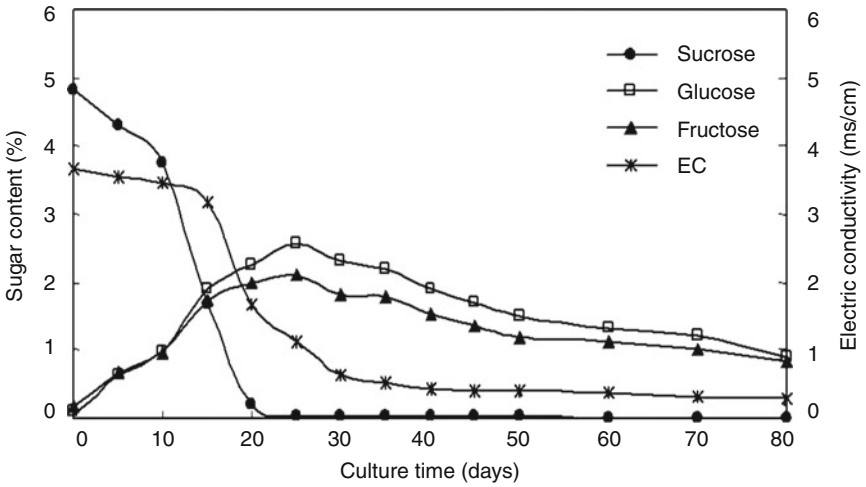

Fig. 20.10 *Panax ginseng* adventitious root cultures: concentrations of residual sugar (*circles*, sugars; *rhombuses*, glucose; *triangles*, fructose; *inverted triangles*, electrical conductivity)

Table 20.9 Effect of medium replenishment on ginsenoside production following 50 days of culturing on ginseng adventitious roots in 5 L balloon type bubble bioreactor containing 4 L of 1.5 strength MS medium

Medium strength and replenishment schedule	Dry weight (g L^{-1})	Ginsenoside content (mg g^{-1} DW)
Control	16.32 ± 0.54	4.17 ± 0.14
Replenishment after 10 days		
0.75 MS	23.49 ± 0.79	4.01 ± 0.16
1.0 MS	24.72 ± 0.51	4.27 ± 0.21
Replenishment after 20 days		
0.75 MS	24.03 ± 0.50	4.94 ± 0.17
1.0 MS	28.66 ± 0.70	4.92 ± 0.15

Each value within the columns represents mean of three replicates ± S.E.

reported in adventitious root cultures of *Echinacea purpurea* [122], cell cultures of *Lithospermum erythrorhizon* [123] and cell suspension cultures of *Taxus chinensis* [124]. Based on the investigation on initial sucrose effect on the cell growth and ginseng saponin biosynthesis, a sugar feeding strategy was formulated to enhance the saponin accumulation by *P. notoginseng* cells [26]. The highest production of crude saponins of 1.77 g L^{-1} (on day 26) was obtained by intermittent sugar feeding during cultivation; the production and productivity of ginseng saponin increased 2.3- and 2.1-fold compared with those of control, respectively.

A major disadvantage of batch processes is that significant amount of time is taken up for system and media sterilization, filling and emptying and cleaning the system. Thus, to improve the cost effectiveness of culturing plant cells, various operational modes including fed-batch, repeated fed-batch, semi-continuous and continuous cultivation have been developed by biochemical engineers and information on these aspects are well documented [93, 97]. The fed-batch operation involves the addition of one or more nutrients continuously or intermittently to the initial medium after the initiation of cultivation or at the stage of the batch process. Continuous cultivation includes variants without feedback control (e.g. in chemostats, where the substrate is fed at a constant rate) and with feedback control (e.g. in trubidostats, where the turbidity of the culture is kept constant by adjusting the rate at which substrate is fed, and auxostats, where the pH or dissolved oxygen of the medium is maintained at the set value). Perfusion cultivation is carried out by continuously feeding fresh medium to the bioreactor and constantly removing the cell-free medium while retaining the biomass in the reactor.

20.8 Precursor Feeding

Many plant cell cultures are also used to convert precursors into products by utilizing enzyme systems present in them. The addition of loganin, tryptophan and tryptamine enhanced the production of secologanin [125] and indole alkaloids [126] by *Catharanthus roseus* suspension cultures. Similarly, phenylalanine feeding improved accumulation of paclitaxel in *Taxus cuspidata* [127], and cholesterol feeding influenced the production of conessine in *Holarrhena antidysenterica* [128] cell cultures. For effective precursor feeding, factors such as the concentration of the precursor and the time of addition should be considered when applying it to the cell culture medium.

20.9 Permeabilization

Plant secondary metabolites formed by plant cell cultures are usually stored in the vacuoles and it is, therefore, desirable to extract the products into the culture medium such that the purification procedure may become easier and continuous recovery

and production of the product may be conducted. Removal of secondary metabolites from the vacuoles of the cells would also reduce the possible product inhibition thus increasing the productivity. Many attempts have been made to permeabilize the plant cell membranes in a reversible manner with organic solvents. Organic solvents such as isopropanol, dimethylsulfoxide (DMSO) and polysaccharides like chitoson have been used as permeabilizing agents [129–131]. Hexadecane, decanol and dibutylphthanlate are used for paclitaxol permeabilizaiton in *Taxus chinensis* [124]. However, when various chemicals are used as pemeabilizing agents they affect the cell viability. Therefore, selection of chemical agent with due consideration to its effect on cell growth may lead to substantial release of secondary metabolites. Other permeabilization methods such as electric field stress [132] and ultrasound methods [133] have also been used for recovery of secondary metabolites.

20.10 Immobilization

Immobilization of plant cells with a suitable matrix has been followed to overcome the problems of low shear resistance and the tendency for cell aggregation [4]. The advantages of immobilization include: (1) the extended viability of cells in stationary stage, enabling maintenance of biomass over a prolonged time period; (2) simplified downstream processing (if products are secretory); (3) high cell density within relatively small bioreactors showing reduced cost and risk of contamination (4) reduced shear stress (5) increased product accumulation (6) flow-through reactors to enable greater flow rates and (7) minimization of fluid viscosity which in cell suspensions causes mixing and aeration problems [134]. There are two major methods for cell immobilization: (a) gel entrapment and (b) surface immobilization. The widely used technique for immobilization involves the entrapment of cells in a specific gel or combination of gels, which polymerize around the cells. Calcium alginate is more widely used matrix, other than this, agar, agarose, gelatin, carrageenan and polyacrylamide have also been used [2, 4, 135]. The matrix used for cell entrapment should be non toxic to cells, should show good polymerization activity and it should be cheaper. Immobilization in *Morinda citrifolia*, *Digitalis purpurea* and *Catharanthus roseus* cultures was first reported by Brodelius et al. [136]. Surface immobilization is another method which takes advantage of the propensity of cultured plant cells to adhere to inert surfaces immersed in the liquid. DiCosmo et al. [137] have reviewed the work on plant cell adsorption to surfaces and immobilization on glass fibers. The surface immobilization of cultured cells in *Catharathus roseus*, *Nicotiana tabacum* and *Glycine max* has been reported for the production of metabolites [138, 139].

Some of the reports which showed dramatic effects of immobilization of cells for secondary metabolite production in plant cell cultures are: 100-fold increment in capsaicin production from immobilized cells with foam and gel [33, 140], 13 and 3.4-fold increment in methylxanthin and ajmalicine accumulation from gel immobilized cells of *Coffea arabica* and *Catharanthus roseus* respectively [138, 141].

Search for new biological and synthetic polymers is an extended research now and some immobilization strategies have been identified to increase bioproduction of secondary metabolites in plant cells [142].

20.11 Selective Adsorption of Plant Metabolites/ Two-Phase Systems

A low accumulation level of secondary metabolites in cell cultures in a number of instances may not be due to lack of key biosynthetic enzymes but rather due to feedback inhibition, enzymatic or non-enzymatic degradation of the product in the medium or volatility of compounds produced. In such cases, it is necessary to develop a separation technique which can concentrate the product. For *in situ* product separation of plant cell cultures, liquid-solid culture systems ('two-phase systems') for plant cells consisting of an aqueous nutrient phase and solid polar adsorbents have been preferred because many plant cells are expected to be of a polar character and bind weakly in the lipophilic phase of liquid-liquid systems. The removal and sequestering of the product in a non-biological compartment may increase total production of secondary compounds [143]. Polycarboxylic ester resin, neutral polymeric resin – XAD-7 could absorb berberine, a secondary metabolite from immobilized (alginate trapped) *Thalictrum rugosum* cells [144]. The advantages of adsorbents are that they can be used in bioreactor operation and allow easy separation of adsorbents from cells for the repeated use of cells and adsorbents [144, 145].

Activated charcoal, RP-8 (lipophilic carrier), Zeolith, XAD-2, XAD-4, XAD-7 (XAD is a neutral resin and ion exchanger), polyethelene glycol, β-cyclodextrin, polydimethylsiloxan, wofatite have been tested and used successfully for separation of secondary metabolites in cell suspension cultures of several systems [4]. Among all these, Ambrilte XAD-7 was efficiently used for adsorption and overproduction of paclitaxol from suspension cultures of *Taxus* [146], anthraquinones from suspension cultures of *Rubia akane* [147], and triptolide from adventitious root cultures of *Tripterygium wilfordi* [148].

20.12 Biotransformation

Biotransformation is a process of regio-selective and stereospecific chemical transformation that is catalyzed by the biological systems or entrapped enzymes or permeabilized cells [149, 150]. Biotransformation is another strategy followed for the production of high value metabolites using plant cell and organ cultures. Reactions carried out by such cultures include hydroxylation, glycosylation, glucosylation, oxidoreduction, hydrogenation, hydrolysis, methylations, acetylations, isomerization and esterification of various substrates [149].

Even though, plant cell cultures have high biochemical potential for the production of specific secondary metabolites, sometimes their desired products are not accumulated due to certain metabolic reasons. However, such cultures may retain an ability to transform exogenous substrates into products of interest. The chemical compounds, which can undergo biotransformation mediated by plant enzymes, are varied in nature which includes aromatic, steroid, alkaloid, coumarin, terpenoid, lignin and other molecular species. It is not always necessary for the compounds to be natural intermediates of plant metabolism but even substrate may be of synthetic origin. Plant cell cultures and enzymes have the potential to transform cheap and plentiful substances, such as industrial byproducts, intro rate and expensive products. For example, podophyllotoxin, a precursor of a semisynthetic anticancer drug is generally extracted from its source plant *Podophyllum* species. Kutnye [151] demonstrated that a cell line of *P. paltaum*, active in the biosynthesis of podophyllotoxin, was able to maintain repeated biotransformation of butanolide to the podophyllotoxin analogue. Ramachandra Rao and Ravishankar [152] used freely suspended and immobilized cells of *Capsicum frutescens* for conversion of protocatechuic aldehyde and caffeic acids to vanillin and capcinin. Li et al. [153] used ginseng cultured cells and roots for bioconversion of paeonol into its glycosides that have the radical scavenging effects.

20.13 Organ Cultures as a Source of Secondary Metabolites

Production of secondary metabolites by cell suspension culture is not always satisfactory, and organ cultures such as root, embryo and shoot culture methods have been developed in various plant species as an alternative for the production of secondary metabolites [44, 91, 92, 154]. Shoot cultures have been established in many medicinal plants which can accumulate secondary metabolites higher than that of natural plants. For example, shoot cultures were established in *Bacopa monnieri* for the production of bacoside A and regenerated shoots possessed threefold higher bacoside A than field grown plants [155]. Similarly, the shoots of *Nothapodytes nimmoniana* which were regenerated in the semisolid and liquid medium had several fold higher camptothecin compared to the mother plants (Fig. 20.11) [156]. Hairy roots can be obtained by transformation with *Agrobacterium rhizogenes*, which can grow with or without the supplementation of growth hormones, and have a growth rate, which is similar to cell suspension cultures [44]. Also these hairy root cultures are good producers of secondary metabolites, for example the terpenoid compound withanolide A was produced in optimum quantity in hairy root cultures [157]. The hairy roots were having high multiplication capability and contained withanolide A 2.7-fold higher than non-transformed roots (Table 20.10). Natural adventitious roots are induced in many medicinal plants and are cultivated in flask scale to bioreactors for the production various bioactive compounds [91, 92]. Adventitious root cultures of *Morinda citrifolia* grown in bioreactors showed several fold increment in anthraquinone content compared to field grown or plants grown in greenhouse [92].

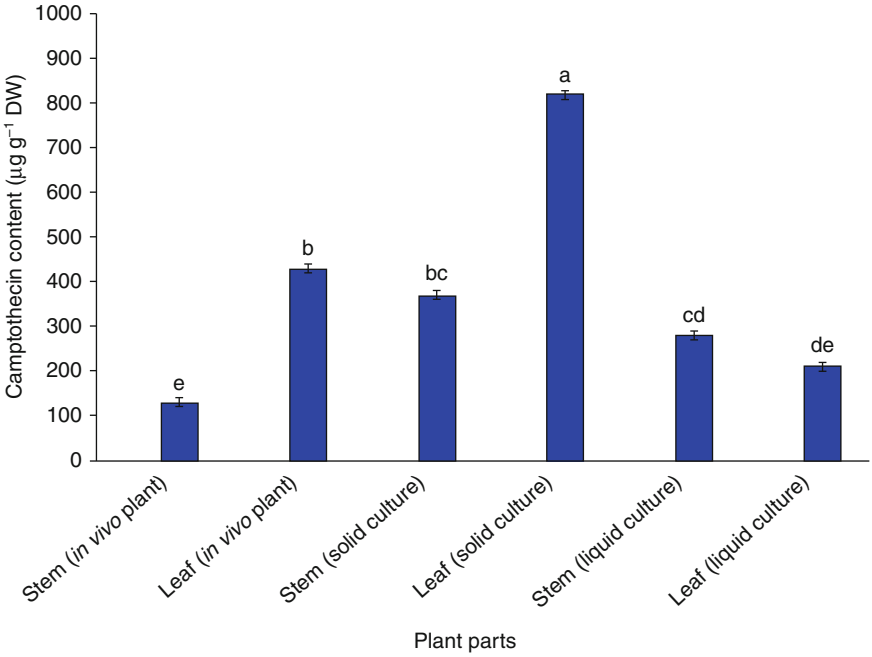

Fig. 20.11 Camptothecin content in plants regenerated on MS semisolid and liquid medium supplemented with or without cytokinins and control plants. *Bars* represent mean ± S.E. Mean values marked with *different letters* are significantly different ($P \leq 0.05$) according to Duncan's multiple range test

Table 20.10 Growth and withanolide content of transformed root lines in MS-based liquid medium after 28 days of culture

Hairy root lines	Doubling time (d)	Dry weight (mg)	Withanolide A (µg g^{-1} DW)
Control (non transformed roots)	60.0	120 ± 1.4 c	57.9 ± 1.9 c
Line 1	8.9	580 ± 2.1 a	148.0 ± 2.6 b
Line 2	13.9	520 ± 2.6 ab	155.6 ± 1.8 a
Line 3	9.8	600 ± 3.0 a	157.4 ± 2.0 a
Line 4	14.6	480 ± 3.5 b	146.5 ± 1.5 b

Values represent mean ± S.E.; $n = 5$ throughout. Values followed by different letters are significantly different ($P \leq 0.05$ Duncan's multiple range test)

20.14 Scale-Up of Plant Cell and Organ Cultures

Plant cells show unique characteristics such as less stability in productivity, higher shear sensitivity, slow growth rate and low oxygen requirements. A wide variety of bioreactor designs have been tested and used for plant cell cultures. Stirred tank reactors, airlift reactors and bubble column reactors for cultivation of plant cells are simply extensions of microbial culture systems with some modifications [154],

and the world's largest plant cell culture facility which was established in Germany (up to 75,000 L) is based on stirred tank models [97]. Centrifugal impeller bioreactor based on the principle of a centrifugal pump has been developed by Wang and Zhong [94, 95] especially for shear sensitive systems, such as culturing plant cells with high shear sensitivity. Successful scale-up of *Azadirachta indica* suspension cultures was developed in stirred tank reactors equipped with centrifugal impeller for the production of azadirachtin [158]. Scale-up of high-density cultivation of *Panax notoginseng* cells in a novel centrifugal impeller bioreactor (CIB) was demonstrated, in which initial k_La was identified to be a key factor affecting cell growth and production of ginseng saponin and polysaccharide [159]. Based on initial k_La level, the CIB high-cell-density cultivation process was successfully scaled up from 3 to 30 L in laboratory. A maximum dry cell weight (DW) and production titer of ginseng saponin and polysaccharide in a 30-L CIB reached 25.5, 1.7, and 2.9 g L^{-1} (on day 15) at an initial k_La value of 28.7 h^{-1}, respectively. Furthermore, by adopting a fed-batch cultivation strategy, a maximum DW and concentrations of total saponin and polysaccharide in the 30-L CIB were enhanced to 30.3, 2.1, and 3.5 g L^{-1}, respectively. The work suggests that the CIB may have great potential in large-scale high-density plant cell cultures for efficient production of useful secondary metabolites [159].

Mechanically driven 'wave reactors' have been recently developed for high shear-stress sensitive plant cells by Ebil and Ebil [160] and absence of air bubbles and wall growth as well as reduced foaming seems to make these reactors suitable for cultivating plant cell and organs [161]. Another reactor called 'slug bubble reactor' consists of vertical, flexible plastic cylinder in which aeration is achieved *via* the generation of large cylindrical bubbles, which move from the bottom to the top of reactors again for useful for cultivation of plant cells which are high-stress sensitive [162].

Dornenburg and Knorr [4] summarized the advantage and disadvantages of few standard bioreactor systems and airlift bioreactors seemed to be ideal for some plant cell cultures which are not highly shear sensitive. Further, airlift bioreactors which spread the air from the base of the reactor through sparger are suitable for cultivation of hairy roots and adventitious roots of various medicinal plants. They are also suitable for scale-up and pilot scale cultivation. Inoculation of 500 g fresh weight adventitious roots of ginseng into 500 L balloon type bubble bioreactors can produce 74.8 kg of root biomass after 8 weeks of culture. The saponin content obtained in small-scale (20 L) to pilot scale (500 L) bioreactors was 1 % based on dry weight [163]. These experimental results have led to the establishment of pilot and plant scale bioreactors (up to 10,000 L; Fig. 20.12) for obtaining ginseng adventitious root biomass and production of ginsenosides for commercial exploitation.

During the scale-up of plant cell and organ cultures, oxygen supply is generally very important as mentioned above. Partial pressure of oxygen may also be critical for secondary metabolite production as shown in high density cell cultures of *Panax notoginseng* [164]. Furthermore, minor gas composition like ethylene and carbon dioxide was identified as a key factor for scaling-up the suspension culture of *Taxus chinensis* for production of taxane diterpene [165]. Other important scale-up factors include shear force and mixing time, as demonstrated in suspension cultures of *Perilla frutescens* [166] and *Taxus chinensis* [167], respectively.

Fig. 20.12 Large-scale (10,000 L) bioreactors developed for cultivating adventitious roots of ginseng

20.15 Conclusions and Perspectives

Plant cell and organ cultures are promising techniques for the production of valuable secondary metabolites which have pharmaceutical, nutriceuticals and industrial importance. This technology is even more attractive with advanced biotechnology approaches such as signal transduction engineering for highly induced biosynthesis of specific targeted products among various heterogeneous metabolites with similar chemical structures but very different bioactivities [168–170]. The recent developments in plant tissue culture techniques and bio-processing have shown promising results to improve biomass growth and the productivity by several folds. Optimization of medium ingredients and culture environmental factors are the basic approaches which should be dealt with individual plant species at flask scale level in the first stage. Various other parameters such as inoculum density, agitation/aeration, elicitation, nutrient feeding, precursor feeding, permealization, and immobilization should be worked out in small scale bioreactor cultures. Care should be taken for the selection of bioreactor types and application of bioprocess parameters at this stage. Adoption of organ culture techniques and scale-up process can lead to significant enhancement in productivity of secondary metabolites. Proper understanding and rigorous analysis of these strategies (Fig. 20.13) would pave the way towards successful commercialization of plant cell bioprocesses.

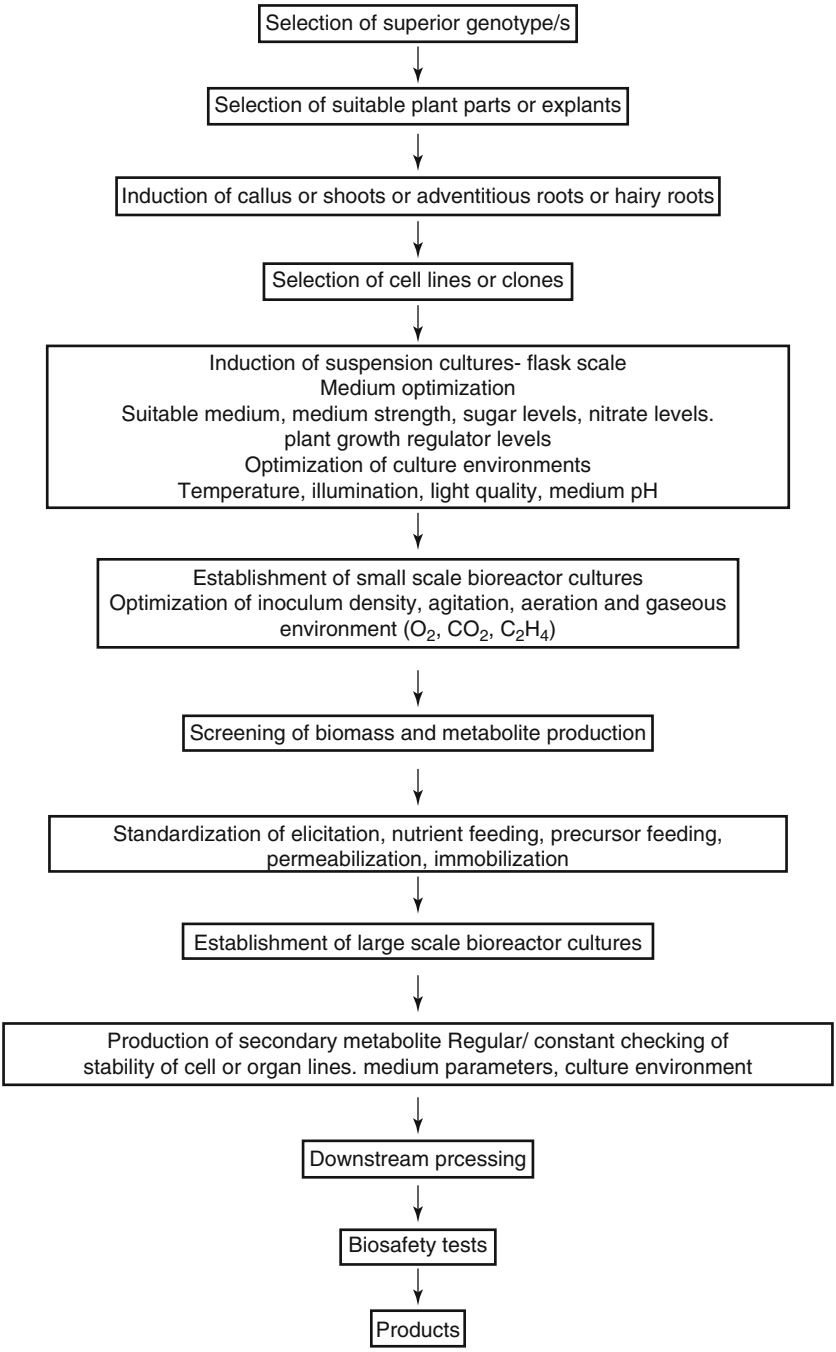

Fig. 20.13 Flow chart of general strategies followed for production of secondary metabolites from plant cell and organ cultures

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