Chapter 8
Plant Cell-Based Bioprocessing

R. Eibl and D. Eibl

Abstract Plant cell-based bioprocessing is the use of plant cell and tissue cultures for the production of biologically active substances (low molecular secondary metabolites and recombinant proteins). The most significant advantage of plant cell culture over the traditionally grown *whole wild plant* or engineered *transgenic plant* is the sterile production of metabolites under defined controlled conditions independent of climatic changes and soil conditions, which means that variations in product yield and quality can be better avoided. Furthermore, regulatory requirements such as the cGMP standards, which have to be adhered to in the early stages of pharmaceutical production, are more easily met.

Moreover, plant cells are capable of performing complex posttranslational processing, which is a precondition for heterologous protein expression. When compared with mammalian cells, which currently dominate in the commercial protein manufacture, plant cell cultures as alternative expression systems guarantee safer processes because there is a lower risk of contamination by mammalian viruses, pathogens, and toxins. In addition to this considerable advantage, the process costs can also be substantially reduced. This is due to the fact that plant cell culture medium is very simple in composition and therefore relatively inexpensive.

This chapter provides an overview of culture types, techniques, and suitable bioreactors used to produce secondary metabolites and recombinant proteins in plant cells. We describe plant cell culture basics, discuss key topics relevant to plant cell bioreactor engineering with application examples, and give an overview of approaches to improving productivity of plant cell-based processes.

R. Eibl, D. Eibl
Institute of Biotechnology, Zurich University of Applied Sciences, Department of Life Sciences and Facility Management, Wädenswil, Switzerland
regine.eibl@zhaw.ch

R. Eibl et al., *Cell and Tissue Reaction Engineering: Principles and Practice,*

8.1 Plant Cell Culture Basics

8.1.1 Characteristics of Plant Cells and Culture Conditions

Plant cells are higher eukaryotic systems with the ability to produce secondary metabolites and glycoproteins, which are more similar to their mammalian counterparts than those from bacteria, yeasts, and fungi. Comparable to the typical cell growth processes of microorganisms and animal cells, those of plant cell cultures are ideally represented by a sigmoid curve with lag, exponential, delay, stationary growth, and lethal phases (Endress 1994; Takebe et al. 1971; Zenk et al. 1975). However, plant cells show morphological and chemical totipotency (potential to form all cell types and/or to regenerate a plant). Their large size of maximum 100 µm results in doubling times of several days and corresponding lower growth rates than microbial cells. The robustness of plant cells, which varies with culture species, culture type, and culture age, is moderate and can be attributed to the presence of a relatively inflexible cellulose wall. It has been described that plant cells can be cultivated under reasonable agitation and aeration conditions without significant responses to hydrodynamic stress, such as loss of viability, cell death, or cell lysis. Less dramatic shear-related effects observed include changes in cell morphology, and reductions in metabolite yield and biomass productivity (Kieran et al. 1997).

The plant cell vacuole, which increases in size over the growth phase, is the main site of product accumulation. While plant cell-based secondary metabolites are largely accumulated in the intracellular environment, recombinant proteins are occasionally secreted into the culture medium due to their composition and structure (Fischer et al. 2004; Misawa 1994). Schillberg and Twyman (2004) report that molecules of 20–30 kDa generally pass through plant cell walls and are thereby secreted into the culture medium. Product formation is growth-associated during active cell growth (Kreis et al. 2001; Misawa 1994) or nongrowth associated when cell growth has ceased (Guardiola et al. 1994; Hamill et al. 1986; Mantell and Smith 1983). Indeed, a large cell mass in the correct cell cycle stage has been found to be the most important precondition for a high product yield.

A temperature between 23 and 29 °C and a medium pH between 5.0 and 6.0 represent optimal parameters for plant cell growth (Endress 1994; Fowler 1988). Aeration is also an important factor for the regulation of cell growth and product accumulation and, as the case may be, secretion. Volumetric oxygen requirements of plant cells are drastically lower than those of microbial systems but comparable to those of animal cells. However, plant cell growth and metabolite production can be limited as a consequence of an inadequate oxygen supply (Zhong 2001). Kieran et al. (1997) and Taticek et al. (1994) describe average oxygen uptake rates (OUR) from 1 to 10 mmol L^{-1} h^{-1}. In small scale, the culture containers or flasks are plugged by the use of gas diffusion materials, ensuring oxygen penetration into the culture containers as a result of molecular diffusion. It is clear that this natural diffusion of oxygen is limited and, as culture volume and plant cell mass increase, plant cell growth is inhibited without continuous shaking or forced aeration (0.1–0.5 vvm).

Interestingly, besides the buffering capacity of carbon dioxide, its addition in concentrations of 0.1–5% has been shown to stimulate the cell growth and product formation of some plant cell lines (Bergmann 1967; DiIorio et al. 1992; Fischer et al. 1995; Fowler 1988; Valluri et al. 1991; Weathers and Zobel 1992; Weathers et al. 1997). According to Mirjalili and Linden (1995), ethylene (ppm range) is a further gaseous metabolite proven to be important for cell growth and/or synthesis of metabolites in individual plant cell cultures. The periodic (dark/light cycle of 8 h:16 h) or continuous introduction of light (0.6–10 klux or 80.7–1,345 µmole m^{-2} s^{-1}) with different wavelengths and intensities promotes the culture growth and product synthesis of heterotrophic, photomixotrophic, and photoautotrophic plant cells, whereas animal cells have to be cultivated in the dark as a consequence of their light-sensitive media compounds. Finally, plant cell cultures can be successfully initiated with relatively high cell concentrations (10% of the culture volume) – unlike bacterial or animal cells – to eliminate long lag phases of 120 h or more and to ensure that initial specific growth rates correlate with maximum growth rates (Endress 1994; Than et al. 2004).

8.1.2 Media

The nutrient supply provided by the culture medium is another critical element when culturing plant cells. The constituents of plant cell culture media summarized in Table 8.1 may be seperated into four groups: (1) doubled distilled and deionized water (95%), (2) the basal medium, which consists of the carbon source and organic as well as inorganic supplements, (3) the phytohormones or growth regulators, and (4) the support matrix such as agar, agarose, or gellan gums (e.g., Phytagel, Gelrite) if required, as in the case of solidification of the medium (Murashige 1973). About 80% (Evans et al. 1983) of all plant cell cultivations are realized in MS basal (MS standard) medium developed by Murashige and Skoog for tobacco tissue cultures (Murashige and Skoog 1962) and/or their modifications. It differs from other typical plant cell culture basal media, such as B5 medium established by Gamborg et al. (1968), in its very high concentration of nitrate, potassium, and ammonia. Plant cell growth and corresponding product formation are strongly influenced by modifications in carbon source, phosphate level, nitrate-to-ammonium ratio, carbon-to-nitrogen ratio, and phytohormones. It is therefore common to optimize the medium by modifying one or two kinds of the basal culture media listed by Endress (1994), or by varying phytohormone types and their concentrations (Kreis et al. 2001; Saito and Mizukami 2002).

Generally, sucrose (2–5%) is the most used carbon source for plant cells. Extracellular invertase hydrolyzes sucrose to monosaccharides such as glucose and fructose. During plant cell growth invertase is excreted into the medium, and the monosaccharide concentrations change according to cultivation time. In contrast to phosphate (phosphate anions or potassium dihydrogen phosphate) and nitrogen (nitrate anions, ammonium cations, amino acids, or protein hydrolysates), which support

Table 8.1 General composition of plant cell culture media

	Basal medium			Phytohormones (growth regulators)	
Water	Carbon source	Organic supplements	Inorganic supplements		Support matrix
	Sucrose	Amino acids: Ala, Arg, Asn, Cys, His, Ile, Leu, Met, etc.	Microelements in μM concentrations: Fe, Mn, Zn, Cu, Mo, I, B, Co	Auxins: IAA, 2,4-D, NAA, IBA, 2,4,5-T	Agar
	Glucose	Vitamins and cofactors: myo-Inositol, thiamine, pyridoxine, folic acid, ascorbic acid, tocopherol, yeast extracts[a]	Macroelements in mM concentrations: N, S, P, K, Mg, Ca	Cytokinins: kinetin, BAP, zeatin, purine, adenine	Agarose
	Fructose			Coconut milk or coconut water[a]	Gellan gums
	Sorbitol			Gibberellines[a]: GA_3, GA_4, GA_7	
				ABA[a]	

[a] Occasionally used

cell growth and lower product synthesis (Nettleship and Slaytor 1974; Do and Cormier 1991), an increase in the initial concentration of sucrose can lead to an improved product concentration, as described by Petersen et al. (1992). However, opposite results have been reported by Oksman-Caldentey et al. (1994) and Chattopadhyay et al. (2003), who showed that maximum product content was obtained with a lower medium sucrose concentration than that required for maximum plant cell growth. They speculated that high sugar concentrations may result in plasmolysis of the plant cells and lead to decreased biosynthesis of secondary metabolites. Furthermore, physiological studies indicate that ammonium is generally used before nitrate in plant cells (Hilton and Wilson 1995; Wilhelmson et al. 2006).

Growth regulators decisively affect the growth process through the ratio of auxins, such as indole-3-acetic acid (IAA) or its most common alternatives 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), to cytokinins, such as kinetin, benzylaminopurine (BAP), zeatin, purine, and adenine. While low auxin and high cytokinin concentrations typically stimulate cell growth, high auxin and low

cytokinin concentrations promote cell division. In fact, it is important to select the most appropriate phytohormones (as they differ in effect) and to determine their optimal concentration by considering the desired tissue or cell culture type. For example, 2,4-D used as a dedifferentiating hormone in higher concentrations supports rapid callus induction, but its application may cause severe growth abnormalities or even result in culture mutations during long-term cultivations. It also often inhibits the regeneration process (Hess 1992). Recombinant protein production and secretion is influenced by supplements that stabilize the proteins (Sect. 8.4), such as polyvinylpyrrolidone, gelatin, bovine serum albumin, and sodium chloride added to the frequently used standard MS- or B5- medium (Shadwick and Doran 2004). Some attention should also be paid to medium sterility (which is achieved by classical standard- or overkill-sterilization treatment in autoclaves (121 °C, 15–20 min) or sterile filtration with 0.1 or 0.2-μm filters) as heat-sensitive constituents are contained in the Newtonian fluid-like culture medium.

8.1.3 Plant Cell Culture Types and Their In Vitro Initiation

8.1.3.1 Callus Cultures

In the in vitro culture of plant cells, the induction of callus is a fundamental step. The term callus describes an amorphous mass of unorganized parenchyma cells formed by the proliferation of plant cells as a protective response at the surface of explant tissue cut with a sterile scalpel. In this process, sterile organs or pieces of tissue such as seeds, embryos, stem sections, leaf- or root- pieces are referred to as explants. Achieving sterility of the plant material requires surface sterilization with chemical agents to kill bacteria and fungi living on the plants while ensuring minimum damage to the treated tissue. Solutions of calcium hypochlorite and sodium hypochlorite in concentrations of 0.5 up to 10% have usually proved to be the most suitable during exposure times of between 5 and 30 min (Bhojwani and Razdan 1983; Endress 1994). A further cheap, ready-made sterilant is a 5–7% solution of the toilet disinfectant "Domestos." The three-stage sterilization process depicted in Table 8.2 has become the accepted rule. For lignified plant material, such as stem tissues or leaves with a waxy surface, an additional ultrasonic treatment is required (Bentebibel 2003).

The formation of the callus is usually achieved by placing the explant on an appropriate solid growth medium with phytohormones in a closed Petri dish incubated at 25 °C in darkness or low light. The running process of cell dedifferentiation is characterized by cell division as well as by elongation and changes in metabolic activity.

After a period of 3–6 weeks, the primary callus formed is normally transferred to a fresh medium after sterile elimination of necrotic parts, which are brown in color (attributed to oxidation of phenolic ingredients). To ensure callus growth, the inoculum should be of uniform size and sufficiently large. As Endress (1994) outlined,

a diameter of 5–10 mm for 20–100 mg fresh weight should be provided. Rapid growth with doubling times of between 7 and 10 days and a white, light yellow, green, or red color generally indicate a healthy callus culture. Besides color, two different types of callus can be distinguished: the friable callus (crumbled or fragmented) and the lignified callus (nonfriable callus). Figure 8.1 shows cells of friable callus of *Vitis vinifera* vs. Uva Italia induced from berry skin. These cells were grown on standard MS medium with additives (Calderón et al. 1994) and maintained at 25 °C in the dark.

The successful establishment of a callus culture and its appearance depend on the donor tissue, the surface sterilization method, the culture conditions, the age of the callus (aging of callus can be characterized by a growing number of lignified cells), and the medium. Sometimes it is advisable to optimize the callus medium by varying auxin and cytokinin concentrations and to work with a callus induction and callus growth medium separately (Endress 1994; Evans et al. 2003; Hess 1992). Above all, it is well known that callus cultures are predisposed to genetic instability

Table 8.2 General sterilization procedure for plant fragments

Stage of procedure	Description
Presterilization (1)	Scrub carefully, clean or rinse under running tap water
	Rinse or submerge briefly in absolute ethanol
Sterilization (2)	Submerge for 5–30 min in 0.5–10% sodium or calcium hypochlorite
Poststerilization (3)	Wash three times with sterile water
	Dry with sterile tissue paper

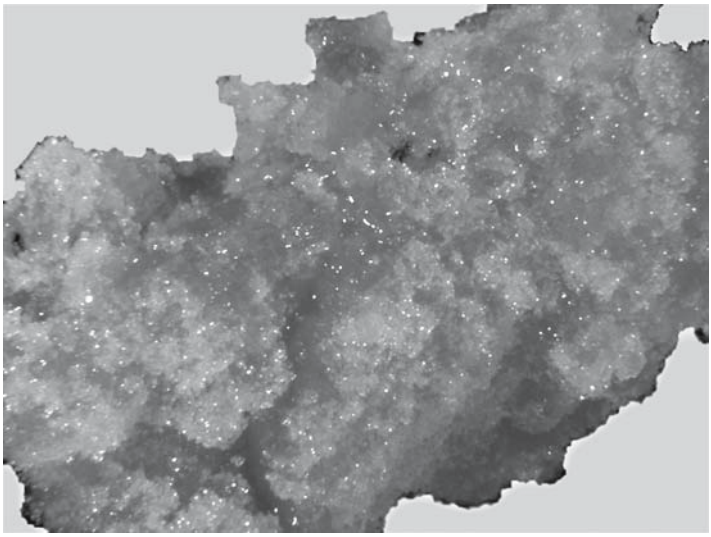

Fig. 8.1 Cells of friable callus of *Vitis vinifera* vs. Uva Italia (established by S. Cuperus, Zurich University of Applied Sciences, Switzerland)

and loss of their morphogenetic characteristics during long passage (subculture) periods. Therefore, it is recommended that callus cultures are passaged every 3–4 weeks depending on the species and the growth rate. The important steps in callus induction and propagation are schematically illustrated in Fig. 8.2.

8.1.3.2 Plant Cell Suspension Cultures

To initiate plant cell suspension cultures, the callus produced is dispersed by inoculating the fragments into a liquid culture medium (Fig. 8.2). The callus friability and its inoculation rate (50–100 g L^{-1} fresh weight) are key issues in this process. To obtain friable callus, Evans et al. (2003) describe a number of procedures including the application of a special callus medium with rising auxin concentration, transfer in liquid medium, and repeated passages in shake flasks (25 °C, 100 rpm) until the callus reaches friability, as well as the addition of pectinase to nonfriable callus growing in liquid medium. After the initial passage, which includes cells breaking off from the friable callus and a suspension beginning to form, the culture is usually filtered with a sieve (300–500 µm) to remove larger aggregates and is finally subcultured to fresh medium in a ratio of 1:1.

This whole procedure (Fig. 8.2) is called homogenization. It aims to produce a homogeneous suspension and is repeated over 2–4 passages. Established plant cell suspension cultures are generally maintained in shake flasks (100 mL flasks with 20 mL medium) at 25 °C and between 100 and 120 rpm, and serially subcultured in the late exponential phase. This is performed every 7–10 days for fast-growing cells that reach high cell masses, and every 14–21 days for slower growing cells characterized by moderate or low cell masses. It is obvious that plant cell suspension cultures grow faster than their callus cultures. Doubling times of between 0.6 and 5 days along with growth rates of 0.24–1.1 d^{-1} are reported in the literature (Hess 1992). Maximum biomass concentrations typically range between 10 and 18 g dw L^{-1} or 200 and 350 g fw L^{-1} (James and Lee 2001).

Plant cell suspension cultures display a high degree of culture heterogeneity as well as variability in terms of cell morphology (including cell size, cell shape, and cell aggregation), rheological characteristics, growth, and metabolic pattern (Hall and Yeoman 1987; Hess 1992; Yanpaisan et al. 1998, 1999). The changes are mainly associated with the chemical and hydrodynamic environment of the cells (Kieran et al. 1997, 2000). The size of single plant cells is typically in the range of 10–100 µm. The most common shapes of plant cells in suspension are the spherical morphology (e.g., the majority of carrot suspension cells, taxus suspension cells, and grape suspension cells) and the rod morphology (e.g., most tobacco suspension cells). Lengthy subculture intervals may slow down cell division, activate cell elongation (which occurs after cell division), and result in changed cell morphology. In this case, a change from an original spherical shape to an elongated shape may occur (Curtis and Emery 1993).

Generally speaking, plant suspensions cells very rarely grow as single cells. They form a few large (or even huge) aggregates, which reach many millimeters

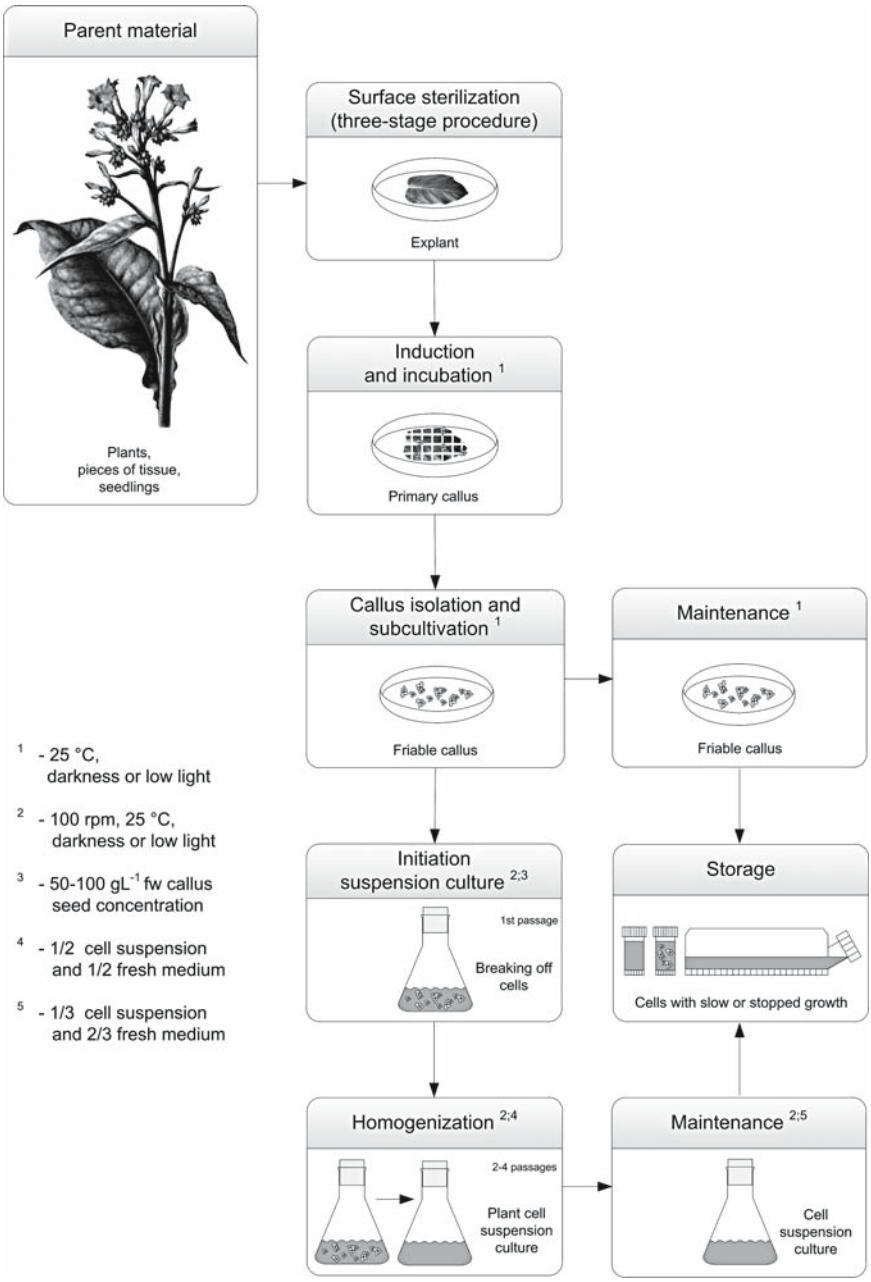

Fig. 8.2 Schematic representation of the procedure for plant suspension culture establishment and maintenance

in diameter and consist of hundreds of highly mitotic and less mitotic cells. The aggregation (clumping) is based on cell adhesion and results from the secretion of cell wall extracellular polysaccharides, which prevent cell separation after cell division (Glicklis et al. 1998). Chattopadhyay et al. (2002) reported an enhancement of cell clumping especially in the later growth stages. While cell aggregation is viewed as desirable for secondary metabolite production due to its promoting of cellular organization and differentiation, this feature may involve oxygen or nutrient gradients, which complicate the cultivation procedure. For plant cell suspension cultures, the critical dissolved oxygen level, below which growth is seen as a result of oxygen limitation, has been commonly quoted as ~15–20% air saturation (Kieran et al. 1997; Yu et al. 1997).

In addition, it appears that cell elongation and aggregation can influence rheological properties of plant cell suspension cultures. Most plant cell lines growing to low or moderate cell concentrations behave like a Newtonian fluid. Non-Newtonian behavior (Bingham plastics characteristics, pseudoplastics characteristics, Casson fluids) associated with higher culture viscosity has been described for high plant cell concentrations (high biomass concentrations, high cell density cultures) and elongated, filamentous cells entangled in a network of cells (Curtis and Emery 1993; Su 2006). Finally, it should be mentioned that suspension cultures often tend to be unstable and unproductive over time (Deus-Neumann and Zenk 1984). Possible explanations for this phenomenon are genotypic and phenotypic variations (i.e., somaclonal variations: changes in chromosome numbers, chromosome structure, and DNA sequence) during cultivation time. Nevertheless, suspension cultures are the most used plant cell culture type in the research and production of secondary metabolites and r-proteins.

8.1.3.3 Hairy Root Cultures

Hairy roots (or transformed roots, as they are more accurately called) are generated by the transformation of plants or explants with agropine- and mannopine-type strains (A4, ATCC, 15834, TR7, TR101, etc.) of *Agrobacterium rhizogenes*, a Gram-negative soil bacterium. When the bacterium infects the plant or explant, the T-DNA, that is, transfer DNA originating from root-inducing plasmid (Ri plasmid), is transferred and integrated into the genome of the host plant. This transformation process produces two by-products: hairy roots and opines (transferred genes, which have been involved in hairy root formation, including genes leading to opine production) (Chilton et al. 1982). In most cases, wounding a sterile leaf (midrib and major veins) or stem tissue is carried out with the sterile tip of a needle attached to a syringe before infection takes place. Two main infection methods are reported in the literature. In method 1, which is most suitable for leaf explants, one to two drops of fresh (two days old) undiluted bacterial suspension is transferred to each wound site (Hamill and Lidgett 1997). In contrast, method 2 works with liquid diluted infection medium in which the explants (leaves, callus, and seedlings) are

submerged for about 3 min (Hamill and Lidgett 1997; Yoshikawa 1997; Komari et al. 2004; Vervliet et al. 1975).

Cocultivation follows, in which plant cells divide and dedifferentiate, and bacteria divide and infect the wounded plant tissue. The resulting transformed cells acquire the capacity to develop into a root system at the infection site. This system branches out to a greater extent than the ordinary roots of plants, and is covered with tiny multiple root hairs. Generally, cocultivation in Petri dishes or other suitable cultivation containers is accomplished by the incubation of the infected explants for 2–5 days at 25 °C in low light or darkness on solid MS- or B5- medium. After cocultivation, the infected explants are cleared of excessively growing *Agrobacteria* by transferring them to a culture medium, which also contains antibiotics such as carbenicillin, cefotaxime, or ampicillin. Because of the appearance of bacterial infections, repetition of the transfer to fresh medium with antibiotics at intervals of 2–4 days is stringently necessary. The neoplastic hairy roots may be maintained indefinitely in solid or liquid culture by subculturing the root tips as illustrated in Fig. 8.3. During the process of adapting hairy roots growing on solid culture medium to liquid medium, a change in root thickness (thinning) involving more rapid growth is not unusual (Carvalho et al. 1997).

Indeed, it is not surprising that root growth is not homogeneous because the rapidly dividing meristematic cells are restricted to root tips. After the cells stop dividing, they grow firstly by elongating and subsequently by branching, which results in lateral root production. Consequently, Hjortso (1997) distinguished between two types of cells in hairy roots: (1) dividing tip cells in the apical meristem and (2) nondividing cells elongating, differentiating, and representing the bulk of hairy roots. Even in shake flasks, a densely packed root mass with heterogeneous structure and the afore mentioned root hairs play a detrimental role for mass transfer of fluid and oxygen because the root hairs induce fluid flow stagnation and high levels of liquid entrainment as well as oxygen limitation (Bordonaro and Curtis 2000; Shiao and Doran 2000; Yu et al. 1997). In general, hairy roots respond to changes in their cultivation environment. For example, the number of root hairs, root length, and root tip viability can be reduced by drought or shear stress.

A clear indication of excessive shear stress is callus formation at root tips (Yu et al. 1997). On the one hand improved oxygen transfer efficiency increases the formation of root hairs and their length (Hofer 1996; Carvalho et al. 1997). On the other hand, Bates and Lynch (1996) describe an increase in root hair formation under nutrient-limiting conditions. Of course, the morphology of hairy roots is affected by many further factors including the plant species and the *Agrobacterium rhizogenes* strain used in hairy root induction.

Although the morphological character of hairy roots complicates their optimized in vitro cultivation, other properties make hairy roots very attractive for the commercial production of valuable metabolites. A significant advantage of hairy roots is that they are generally easy to isolate and grow in a defined medium. Because auxin metabolism is altered in plant cells after transformation with *Agrobacterium rhizogenes*, the addition of exogenous growth regulators or phytohormones becomes unnecessary. Most importantly, fully differentiated hairy roots have the strong tendency to be genetically and biochemically stable. They show a rapid

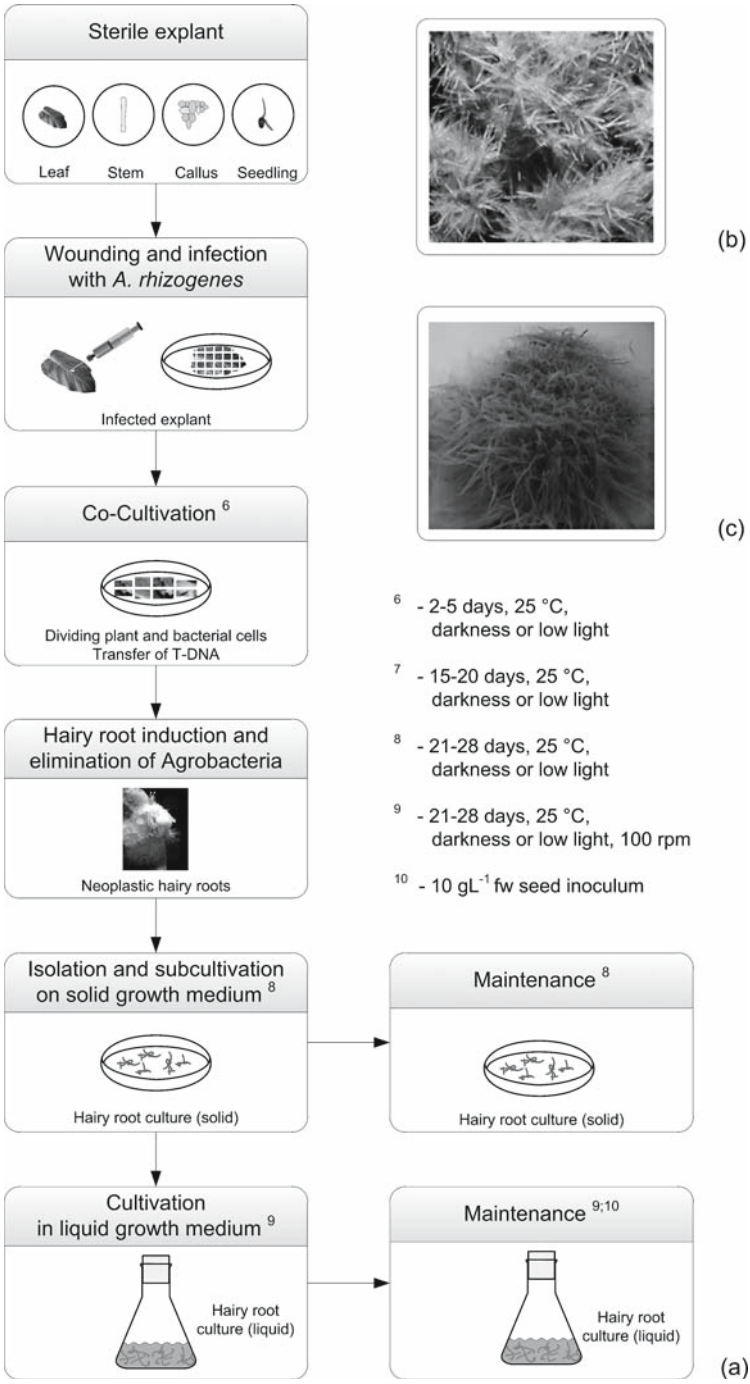

Fig. 8.3 Hairy root cultures: (**a**) Typical techniques to initiate hairy roots on solid and in liquid medium, (**b**) Hairy roots of *Hyoscymus muticus* growing on solid B5 medium

growth with average doubling times resembling those of disorganized cell suspensions. For example, Hess (1992) and Doran (2002) present doubling times of 1 day for hairy root cultures of *Atropa belladonna* and *Nicotiana tabacum*, and 8 days for hairy roots of *Arabidopsis thaliana* and *Solanum aviculare*.

Furthermore, hairy roots have been found to synthesize secondary metabolites at similar or higher levels to those found in whole plants (Flores 1987; Hu and Du 2006; Oksman-Caldentey and Hiltunen 1996; Rhodes et al. 1994; Sevón and Oksman-Caldentey 2002) and to have light-guiding properties (Towler et al. 2006). In addition to their recombinant protein expression ability, transgenic hairy root cultures exhibit a significantly greater long-time stability than transgenic suspension cultures (Sharp and Doran 2001).

8.1.3.4 Embryogenic and Shoot Cultures

Like hairy roots, embryogenic and shoot cultures belong to the group of differentiated organ cultures. But today, embryogenic and shoot cultures are mainly used for micropropagation and plant breeding. For both the in vitro production of secondary metabolites and recombinant proteins, these culture types are of minor importance in contrast to hairy root and plant cell suspension cultures. Therefore, the characteristics and the initiation procedure of embryogenic and shoot cultures is not included here. More information on this topic and on the process of developing embryos from somatic cells and tissue (somatic embryogenesis) is provided by Hess (1992), Endress (1994), Evans et al. (2003), Ducos et al. (2007), Gupta and Ibaraki (2006), and Hvoslef-Eide and Preil (2005).

8.1.4 Routine Working Methods in Plant Cell Cultivation

8.1.4.1 Determination of Plant Cell Growth

To characterize and design bioprocesses based on plant cells other than secondary metabolite production or protein accumulation/secretion and nutrient utilization (e.g., sucrose, glucose), the cell growth must be determined. Six direct and indirect methods, which provide information on plant cell growth, have been reported. These methods involve measurement of biomass accumulation (fresh weight, dry weight), cell mass, and cell number (cell concentration) as well as viability, conductivity, osmolarity, and pH (Endress 1994; Naill and Roberts 2005; Tanaka et al. 1993; Widholm 1972).

Plant cell growth is normally measured either by fresh weight or by dry weight. Its determination relies on population averages and does not take plant cell culture heterogeneity into account. Fresh weight values (expressed as g) are obtained by weighing freshly harvested cells. The increase in fresh weight that occurs is due to both cell growth and expansion (indicated by cell size). The dry weight (expressed

as g) excludes errors caused by endogenous water content and is a more useful tool for biomass quantification of plant cells than fresh weight. For dry weight measurement, a known weight of fresh plant cells is dried in an oven at a temperature of between 50 and 60 °C to the point of constant weight (ca. 24–48 h). Alternatively, fresh cells are lyophilized.

For fine plant cell suspensions, the cell mass can be recorded as packed cell volume (PVC) immediately after gentle centrifugation at low speed. In fact, PCV (expressed as %) is defined as the ratio of the volume occupied by solid matter in the form of plant cells or cell aggregates to the volume of the whole sample (aliquots of about 15 mL). By comparing the packed cell volume to the biomass accumulation, a definite correlation can be determined. A further, less common method of measuring the biomass growth of suspension cultures is manual cell counting using an improved Neubauer-type hemocytometer with a depth of 0.1 mm to determine the number of plant cells per unit volume (expressed as cells mL^{-1}). The cell growth in aggregates and the large size of the plant cells presuppose cell dispersion with chemical agents such as hydrochloric acid, chromic acid (Reinert and Yeoman 1982), EDTA, or pectinase before counting. Nevertheless, if fluorescein diacetate, trypan blue, or Evan's blue is used as a dye for staining suspended cell counts, plant cell viability can be roughly determined (Steward et al. 1999; Widholm 1972). Viability is defined as the ratio of viable cells to total cells, and has values between 0 and 1, that is, between 0 and 100%.

Measurements with a conductivity meter in liquid culture medium readily allow the indirect monitoring of biomass growth. There is an inverse relationship between electrical conductivity (expressed as $mS\ cm^{-1}$) and cell weight. In the case of liquid plant cell cultures, a number of authors have shown that an increase in cell dry weight can be correlated linearly to a decrease in medium conductivity (Bais et al. 2002; Ryu and Lee 1990; Taya et al. 1989a; Than et al. 2004). This is probably caused by an uptake of medium salts in the form of ions by the cultured cells and resulting biomass growth during cultivation. But medium conductivity depends mainly on electrolyte (mineral) concentration and not on the sugars that are major components of the culture medium. Whereas small changes in biomass level are more difficult to estimate by conductivity measurement, relatively wide osmolarity changes correlate well with small changes in biomass of several glucose-consuming plant cell suspensions (Tanaka et al. 1993). Thus, Madhusudhan et al. (1995) recommend osmolarity ($mOsmol\ kg^{-1}$ units) as a sensitive measure of the growth of plant cell cultures with rapid sucrose-hydrolyzing capability.

The pH measurement is routinely made during the cultivation of liquid cultures. A gradual drop in pH to a value around 4 reflects the initial ammonium uptake and acidification caused by cell lysis within 20–48 h, whereas the pH returns to a stable value of about 5 related to the uptake of nitrates after a few days of cultivation (Ziv 2000). If the growth at the single-cell level should be quantified, flow cytometry may be utilized besides traditional measures of cell growth such as biomass accumulation for suspended plant cell populations (Evans et al. 2003; Naill and Roberts 2005; Yanpaisan et al. 1998, 1999). Parameters frequently calculated (specific growth rate and doubling time excepted) to characterize plant cell growth on a biomass basis are summarized in Table 8.3.

Table 8.3 Parameters characterizing plant cell growth on a biomass basis

Parameter	Description and formula	Used for
Fresh biomass concentration (FCM_{tx})	Ratio of cell fresh weight at time tx to initial volume of medium: $FCM_{tx} = fw_{tx}/V_i$ [g fw L^{-1}]	All types
Dry biomass concentration (DCM_{tx})	Ratio of cell dry weight at time tx to initial volume of medium: $DCM_{tx} = dw_{tx}/V_i$ [g dw L^{-1}]	
Dry substance (DS_{tx})	Ratio of cell dry weight at time tx to cell fresh weight at time tx: $DS_{tx} = dw_{tx}/fw_{tx}$ [none]	
Biomass productivity (BMP)	Ratio of cell weight increase to maximum cell weight and the cultivation time during which the maximum cell weight was obtained (FCM_f and DCM_f = maximum values): $BMP_{dw} = (DCM_f - DCM_i)/t$ [g dw L^{-1} d^{-1}]; $BMP_{fw} = (FCM_f - FCM_i)/t$ [g fw L^{-1} d^{-1}]	
Growth index or growth ratio (GI)	Ratio of final (maximum) cell dry weight to initial cell dry weight: $GI = dw_f/dw_i$ [none]	Organ cultures (e.g., hairy roots)

Mathematical models developed for callus and suspension cultures describe cell growth by modified Monod kinetics and reactions of the first order (Guardiola et al. 1994; Val 1993; Xing et al. 2001). To predict increase in biomass of hairy root cultures, branching models (Hjortso 1997; Kim et al. 1995; Taya et al. 1989c) incorporating the important features of hairy root growth (Sect. 8.1.3.3) and a population model (Han et al. 2004) have been proposed. The population balance model shows biomass growth resulting from root elongation caused by both cell division and the formation of new lateral branches. It also shows that the formation of a new branch depends on the age of the parent branch.

8.1.4.2 Genetic Transformation

Nowadays two indirect *Agrobacterium*-mediated transformation methods based on *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* are routinely used to transfer DNA into plant cells of both monocotyledonous and dicotyledonous species. While *A. rhizogenes* causes hairy root disease (Sect. 8.1.3.3), *A. tumefaciens* induces a tumor formation process, commonly known as crown gall disease, via an identical process. Genes involved in this tumor formation accumulate on the tumor-inducing plasmid, Ti plasmid, which encodes the virulence genes *vir* and transfer DNA into the plant genome. The most critical factors in efficient *Agrobacterium*-mediated transformation procedures are the vectors and strains, the type and quality of the starting material, the concentration of the *Agrobacterium* inoculum, the composition of the cocultivation medium, the temperature and pH range of cocultivation,

and finally the antibiotics added to the plant cells to remove the *Agrobacterium* cells (Komari et al. 2004).

A more modern transformation method, developed in the 1980s, is particle bombardment (also called biolistic method, gene gun, or particle gun method), which has no intrinsic limitations with respect to target tissue, species, or genotype as described for *Agrobacterium*-mediated transformation. It remains a direct transformation method in which high-velocity microprojectiles from gold or tungsten breach the cell wall and membrane, and are thus used to introduce engineered DNA into plant cells and tissue of any type (leaves, shoot tips, embryogenic cultures, callus cultures, cell suspensions, whole plants). In this way, the DNA diffuses from the microprojectiles and is then transiently or permanently incorporated into the plant genome (Twyman and Christou 2004). Since the 1990s, particle bombardment has also been used successfully in combination with other transformation techniques (Bidney et al. 1992; Hansen and Chilton 1996; Rasmussen et al. 1994), for example, *Agrobacterium*-mediated transformation. As demonstrated, e.g., for mosses, the longstanding polyethyleneglycol (PEG)-mediated DNA transfer is the method of choice if Agrobacterium-mediated transformation or biolistic transformation is not possible (Gorr and Wagner 2005).

Studies have revealed that transgenic plant cell suspensions can be developed from transgenic callus cultures produced with a particle gun or explants from transformed plants, as well as from *Agrobacterium tumefaciens*-mediated transformation of wild-type suspensions, and the biolistic delivery of plasmid DNA into plant suspension cells. Currently, transgenic hairy roots can be initiated by infecting transgene-containing plants or explants with *A. rhizogenes*, performing root initiation and transformation using engineered *A. rhizogenes* strains containing modified T-DNA, and direct *A. tumefaciens*-mediated transformation of established hairy root cultures from wild-type (Shadwick and Doran 2004). As described for mammalian cells, doubling time and growth rate of plant cells can also be increased by genetic engineering (Su 2006). For more detailed information on the genetic transformation of plant cells, the reader is referred to Christou and Klee (2004) and Fischer and Schillberg (2004).

8.1.4.3 Storage

Plant cell cultures are maintained either in slow-growth storage or, for long-term conservation, in the form of cryopreservation. While preserving all the characteristics of the cells, both methods reduce the frequency of subcultivation and thereby reduce contamination risk, labor, as well as media costs. Slow growth can be achieved by various simple methods including a temperature shift to a range between 2 and 10 °C, a decrease in light (dim light at about 50 lux) or oxygen, the application of growth medium with stabilizers promoting cell survival, or combinations of these methods. According to Schumacher et al. (1994), slow-growth storage for more than 4 months in static tissue culture flasks with growth medium plus 0.01% charcoal and 1.0% gelatin at a reduced temperature of 10 °C in the dark may

be preferable for suspension cultures of *Agrostis tenuis, Nicotiana tabacum, Nicotiana chinensis, Oryza sativa*, and *Solanum marginatum*.

In contrast to slow-growth storage, it is known from animal cell storage that cryopreservation, which finally occurs in liquid nitrogen and at −196 °C, effectively stops all cellular processes. It is achieved by use of cryoprotectant solutions containing DMSO, glycerol, or sucrose in standard culture medium with controlled cooling or dehydration followed by rapid freezing (Withers 1991; Benson et al. 1998). A cooling rate in the range of −0.25 °C min^{-1} to −2.0 °C min^{-1} down to not less than −40 °C maintained for 30–60 min and followed by rapid cooling to liquid nitrogen temperature has proven to be suitable for many plant species and culture types (Moldenhauer 2003). Successful cryopreservation of cell suspension cultures by slow prefreezing is described by Seitz (1987), and Menges and Murray (2004). Vitrification using highly concentrated plant vitrification solution 2 (PVS2) represents another cryopreservation procedure with extended applicability (Kobayashi et al. 2006). In spite of the long list of cryopreserved plant cells, which includes callus cultures, suspension cultures, isolated protoplasts, embryogenic cultures, hairy roots, and shoot tips (Endress 1994; Kartha 1987; Moldenhauer 2003; Yoshimatsu et al. 1996), cryopreservation is still not widely used in processes that use plant cells as production organisms.

8.2 Bioreactors for Plant Cell Cultures

8.2.1 General Considerations

For plant cell suspensions and hairy roots, suitable bioreactors can be roughly divided into three main types according to their continuous phase (Kim et al. 2002a): liquid-phase bioreactors, gas-phase bioreactors, and hybrid bioreactors (Table 8.4). In liquid-phase bioreactors, the plant cells are immersed continuously and oxygen is usually supplied by bubbling air through the culture medium. The term "submerged bioreactor" is also used for liquid-phase reactors. Mechanically driven bioreactors, pneumatically driven bioreactors, and hydraulically driven bioreactors belong to this category (Sects. 8.2.2 and 8.2.3). Because of the low solubility of gases in liquid-phase systems, gas-exchange limitations and insufficient nutrient transfer (as a consequence of gentle mixing to lower hydrodynamic stress) frequently result in growth inhibition (Singh and Curtis 1994).

By using gas-phase reactors such as the spray reactor or the mist reactor, which represent typical emerged bioreactors (Sect. 8.2.3), oxygen transfer limitation can be reduced or even eliminated. Whereas plant cells, especially organ cultures, are exposed to humidified air or other gas mixtures, the medium containing the nutrients is delivered to cells as droplets produced by spray nozzles or ultrasonic transducers. As readily identifiable from the bioreactor name, the droplet size of mist bioreactors is smaller (usually 0.01–10 µm) than the potentially much greater droplet size of

Table 8.4 Plant cell bioreactor categorization

Plant cell bioreactors				
Liquid-phase bioreactor/ submerged bioreactor			Gas-phase bioreactor/ emerged bioreactor	Hybrid bioreactor
Mechanically driven bioreactor	Pneumatically driven bioreactor	Hydraulically driven bioreactor	Trickle bed reactor (droplet reactor or spray reactor)	Combinations of bubble column and gas-phase bioreactor, for example: bubble column-trickle bed
Stirred reactor	Airlift reactor	Radial flow bioreactor	Mist reactor	
Rotating drum reactor	Bubble column Plastic-lined reactor			Bubble column-mist reactor
BioWave	Slug Bubble bioreactor			Wilson Reactor (bubble column-spray reactor)
Wave & Undertow bioreactor				

spray bioreactors (10–10^3 µm). Finally, the hydrodynamic stress is low in gas-phase bioreactors (Towler et al. 2006; Weathers et al. 1999; Whitney 1992; Wilson 1997).

Hybrid bioreactors represent combinations of submerged and emerged bioreactors, such as the famous 500 L Wilson Reactor for hairy roots (Sect. 8.2). They have been developed to circumvent manual loading of the bioreactor growth chamber in order to uniformly distribute the production organisms, for example, roots. The hybrid bioreactor switches from liquid-phase to gas-phase operation after the inoculation, distribution, attachment to immobilization points, and short growth phase of the cells.

Small biomass concentrations of plant cell cultures can be grown in virtually any bioreactor configuration. Excellent plant cell growth with biomass productivity > 1 g dry weight L^{-1} day^{-1} requires an optimized and well-characterized bioreactor configuration. For plant cells tending to high cell density growth and/or aggregate formation, their trouble-free inoculation, transfer, and harvest presuppose specially designed bioreactor elements. For example, transfer pipes, inoculation pipes, harvest pipes, and ports all have to be sufficiently dimensioned. Moreover, inoculation should be performed by application of gravity, pressure, or vacuum, and high shear stress from pumping should be avoided.

Process monitoring and control are generally facilitated by standard bioreactor instrumentation, which uses pressure-, temperature-, pH-, and air sensors as well as gas flow rate-, pO_2- (dissolved oxygen), pCO_2- (dissolved carbon dioxide), and

conductivity sensors. The integration of a gas analyzer allows the oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) to be determined.

As previously mentioned, the yield of bioactive substances is determined both by biomass production and by the level of bioactive substances produced per unit biomass (Sect. 8.1.1). Potentially, the importance of the bioreactor type used lies not in maximum biomass production, but rather in stimulating product formation by guaranteeing an optimal environment (Flores and Curtis 1992; Kim et al. 2002b). Therefore, the bioreactor should be selected and optimized with the cultivation goal in mind (biomass or bioactive substance) taking into account the cell line's specific morphology, rheology, shear tolerance, growth behavior, and metabolism.

8.2.2 Suitable Bioreactors for Plant Cell Suspension Cultures

Cell suspension cultures in general are regarded as the most suitable plant cell culture type for large-scale biotechnological applications and are cultured in mechanically or pneumatically driven aerated submerged bioreactors. Stirred reactors, bubble column reactors, and airlift reactors directly derived from microbial fermenters were initially used with only minor modifications to grow plant suspension cells (Eibl and Eibl 2002). In aerated/submerged plant cell suspension bioreactors, engineering analysis demonstrates that hydrodynamic-related stress adversely affecting the cultivation course is generally a result of the aeration and mixing system, aeration rate, and/or impeller tip speed used (Kieran et al 2000; Su 2006; Zhong 2001).

In most plant cell cultivations, the air is directly introduced via a sparger (ring, pipe, plate, frit) positioned in the lower part of the bioreactor (Eibl and Eibl 2002). According to Präve et al. (1994), such direct aeration guarantees the highest possible aeration efficiency, this being evaluated by volumetric oxygen transfer coefficients, $k_L a$ values, which are above $20\,h^{-1}$ for cell cultures. When pressure, temperature, and medium are fixed, $k_L a$ values of bioreactors are affected by the aeration rate, gas holdup, and size of the bubbles produced by the sparger. The higher the aeration rate, the larger the bubble size, speed between bubbles and cells, collision risk, and damaging ability become. If sensitive cells come into contact with air bubbles, cells cultivated in bubble-aerated bioreactors may be damaged as a consequence of cells and bubbles rising to the surface and subsequent bubble bursting (Storhas 1994). As mentioned in Sect. 8.1.1, typical oxygen demands and the resulting aeration rates for high plant cell growth do not lead to permanent cell damage for most plant cell lines. In general, $k_L a$ values of about $10\,h^{-1}$ are necessary for high plant cell culture growth (Takayama and Akita 2006) in large-scale culture systems. However, Pan et al. (2000) reported a growth stimulating effect of $k_L a$ values above $15\,h^{-1}$ in stirred *Taxus chinensis* suspension cell cultivations.

Another point to consider is that the presence of extracellular polysaccharides, fatty acids, and high sugar concentrations in the plant cell culture medium

combined with bubble aeration promotes foaming at the culture broth surface, which is a serious problem particularly for the pneumatically driven plant cell bioreactors such as airlift and bubble column reactors. The result of extensive foaming is a crust of foam-entrapped cells adhering to the inside of the bioreactor's vessel (called wall growth phenomenon) as well as clogging of the air exhaust filter, which constitutes a contamination risk. Ceramic or sintering steel porous spargers (Kim et al. 1991; Zhong et al. 1993) generating fine bubbles with higher $k_L a$ values, bubble-free aeration via tubes of silicone (Abdullah et al. 2000; Ziv 2000), external aeration (Carvalho and Curtis 1998; Kino-Oka et al. 1999), and indirect aeration (spinfilter, eccentric motion stirrer, cell-lift impeller) (Eibl et al. 1999; Su 1995; Valluri et al. 1991) can be used to overcome the disadvantages of sparger rings to plant cells at high bubble aeration rates. However, with the exception of external aeration and ceramic or sintering steel porous spargers, sparger systems have a limited scale-up potential. It is evident that, as a general rule, direct aeration is more effective and advantageous than indirect medium aeration conducted in the outer loop. Paek et al. (2005) successfully reduced foaming and cell growth on the vessel wall in bubble columns by designing balloon-type bubble bioreactors. Similar effects, including minimized wall growth, can be achieved by oxygen enrichment, which causes low gas flow rates as found in the Plastic-lined bioreactor (Fig. 8.4g) by Curtis for *Hyoscyamus muticus* suspension cells. This low cost bioreactor type has a cultivation bag of plastic film, which is inserted in a bubble column vessel. Its 150 L system (100 L culture volume) produced biomass with over 15 g dw L^{-1} during the 33-day culture period (Curtis 2004).

In investigations carried out in our lab we observed that, in the course of different plant suspension cell cultivations producing stable foam layers, flotation effects increased, which led to rising cell entrapment in the foam layer and nutrient limitations for these cells during long-term cultivations. A reduction of foaming can be accomplished by the addition of antifoam agents such as silicone-based agents, polypropylene glycol antifoam agents, and/or mechanical foam disruption. It should be noted that in plant suspension cell cultivations an overdose of such antifoam agents may reduce oxygen transfer (Kawase and Moo-Young 1990), and that mechanical foam disruption is theoretically possible: unfortunately, applications with hydrocyclones or mechanical turbines do not exist. At high cell mass or biomass concentrations >30 g dw L^{-1}, a further disadvantage of using bubble columns and airlift bioreactors to cultivate plant cell suspension cultures is insufficient mixing. This results in poor oxygen transfer (air bubbles cannot be dispersed satisfactorily $\rightarrow k_L a$ decreases) and heterogeneous biomass distribution. Therefore, these bioreactor types are not recommended for culturing plant cells at high cell densities, the conventional stirred bioreactor modifications predominating in the literature being more suitable (Doran 1993; Tanaka 2000).

For stirred plant cell bioreactors, the aeration system type and arrangement should be taken into account when choosing the impeller system and its pumping mode. To guarantee mass and temperature homogeneity as well as optimal gas dispersion without causing excessive foaming and shear damage to the plant cell suspension, the sparger of the stirred bioreactor (which usually operates below the

flooding point) is located in the flow direction of the impeller. For instance, surface aeration requires the use of an impeller type with downward pumping mode as this allows better oxygen transfer for the increased culture broth viscosities that result from high cell densities. Long-term experience made with mammalian cells has suggested the advantageous combination of upward pumping axial-flow and radial-flow impellers in stirred bioreactors equipped with frits for aeration. We assume that this will be used in plant suspension cell stirred bioreactors in the near future. Until now, impellers considered suitable have included large slow-moving axial flow impellers with low tip speeds of up to a maximum of 2.5 m s^{-1}, such as marine impellers or special pitched blade impellers, and impellers positioned near the vessel wall, for example, spiral stirrer, helical-ribbon impellers, and anchor impellers. Several descriptions of these impeller types for plant suspension cells can be found in the literature (Eibl and Eibl 2002; Joliceur et al. 1992; Kieran et al. 1997).

In addition, alternative impeller systems have been developed and installed in conventional stirred vessels. Prominent examples are the cell-lift impeller and the centrifugal-pump impeller, which function both as a fluid pump and aerator (Kim et al. 1991; Roberts and Shuler 1997; Wang and Zhong 1996a, 1996b; Zhong et al. 1999). For example, a maximum dry cell mass concentration of 26 g dw L^{-1}, biomass productivity of 0.9 g dw L^{-1} d^{-1}, and a ginsenoside productivity of 29 mg L^{-1} d^{-1} were achieved in cultivations of *Panax notoginseng* cells in a centrifugal impeller bioreactor (Zhong 2001). Radial flow impellers, such as the Rushton impeller, achieve high-energy inputs and are therefore suitable for limited applications with plant cell suspensions. In certain cases, the design has been improved, as with concave blades (Eibl and Eibl 2002; Pan et al. 2000; Schiermeyer et al. 2004).

There have been many studies on the development and use of other mechanically driven bioreactors, characterized by their superior performance in terms of suspension homogeneity and low-shear environment. Tanaka et al. (1983), Shibasaki et al. (1992), and Takahashis and Fujita (1991) successfully used a rotating drum bioreactor for the cultivation of suspension cultures of *Catharanthus roseus, Nicotiana tabacum*, and *Lithospermum erythrorhizon*. In comparison to the other bioreactor types described earlier, the rotating drum bioreactor has a significantly larger surface area in relation to volume. Furthermore, in this bioreactor, mass transfer is achieved with comparatively less specific power input and therefore low hydrodynamic shear.

Eibl and Eibl (2006, 2007), Cuperus et al. (2006), and Krüger (2006) have pointed out the excellent biomass growth of tobacco, grape, and apple suspension cells (maximum biomass productivity in the range of 20–40 g fw L^{-1} d^{-1} and doubling times between 2 and 4 days) in the BioWave reactor (Fig. 8.4d) operating with 1 and 10 L culture volume. Furthermore, reduced shearing (indicated by higher viabilities and no significant change in cell morphology) and reduced foaming were found in the disposable BioWave when compared with cultivations in 2 L stirred bioreactors performed in our lab. These observations can be explained by the hydrodynamic characteristics of the cultivation environment and oxygen transport efficiency ensured by the BioWave. The energy input is caused by rocking the platform, which induces a wave in the culture bag. As a consequence of this wave

induced motion, oxygenation and mixing are realized. The surface of the medium is continuously renewed and bubble-free surface aeration takes place. Provided batch mode and cultivation parameters in the BioWave and the applied stirred bioreactor were comparable, we found $k_L a$ values to be more than twice as high in the BioWave. Assuming a constant rocking angle and culture volume, as the rocking rate is increased, BioWave's power input rises until it reaches a stationary value, which may be followed by a slight decrease resulting from the occurring phase shift of the wave. For a 2 L culture bag containing 1 L culture volume, the phase shift took place at a rocking rate greater than 20 rpm (Chap. 5). The latest disposable bioreactor developments, including the Wave & Undertow bioreactor (WU bioreactor) and the Slug Bubble bioreactor (SB bioreactor), were successfully applied to grow tobacco and soya cells expressing isoflavones and monoclonal antibodies (anti-rabies) up to 100 L culture volume (Girard et al. 2006; Terrier et al. 2006). The WU bioreactor is based on the WIM principle like BioWave, while the SB bioreactor represents a bubble column that allows an easy increase of size by using multiple units.

If illumination is required, particular bioreactors made of glass vessels or plastic bags can be used in illuminated rooms or with external illumination lamps (i.e., fluorescent lamps) installed around them. There are only a few reports on and patents (Takayama and Akita 2006) for internal illumination systems made from tubes (Dubuis 1994) and glass fibers, for example, in respect of the 15 L bioreactor with eccentric motion stirrer and integrated illumination cage (Eibl et al. 1999; Eibl and Eibl 2002). As these systems are costly and fail to introduce light efficiently, they have not been commercialized.

Finally, while the majority of transgenic suspension cells that have been used to express recombinant proteins are ideally cultivated in stirred laboratory bioreactors (Hellwig et al. 2004), photoautotrophic moss suspension cells from *Physcomitrella patens*, which secreted 30 mg L^{-1} d^{-1} human vascular endothelial growth factor (VEGF), are cultivated in a tubular bioreactor. This photobioreactor type is well known from microalga cultivation where light is one of the main factors influencing biomass growth and also scale-up (Decker and Reski 2004; Gorr and Wagner 2005).

8.2.3 Suitable Bioreactors for Hairy Roots

From Sect. 8.1.3.3, it will have become clear to the reader that it is more difficult to cultivate hairy root cultures than plant suspension cells in bioreactors. Without design modifications supporting root inoculation, growth, sampling, and harvest, the stirred bioreactor is generally not suitable for hairy roots despite its dominance in biotechnology. Exceptions reported in the literature are the cultivation of *Catharanthus trichophyllus* L. hairy roots (Davioud et al. 1989), hairy root cultures of *Catharanthus roseus* (Nuutila et al. 1994), strawberry (Nuutila et al. 1997), and beetroot (Georgiev et al. 2006). In general, the impeller can damage the roots even at low specific power inputs. To rule out this possibility, a cage or mesh of stainless

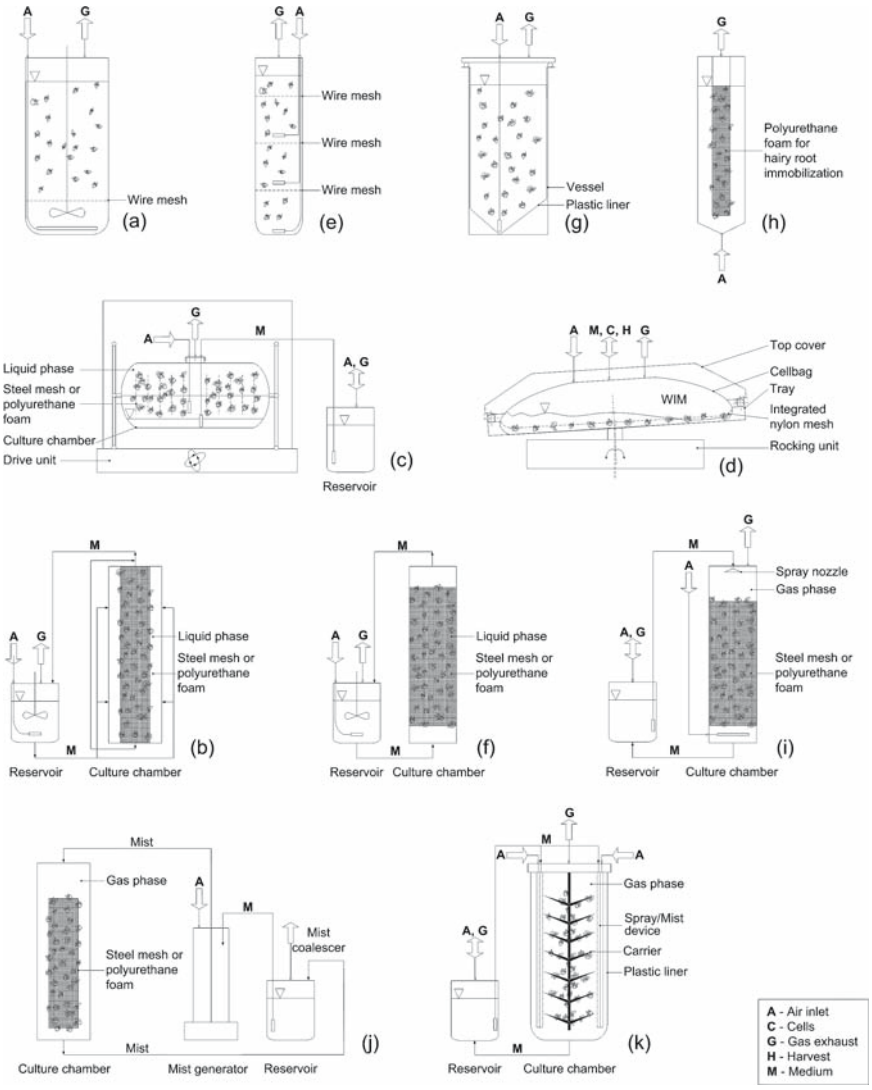

Fig. 8.4 Bioreactors for hairy root cultivation: (**a**) modified stirred bioreactor, (**b**) radial flow bioreactor, (**c**) Inversina, (**d**) BioWave, (**e**) segmented bubble column, (**f**) convective flow bioreactor, (**g**) Plastic-lined bioreactor, (**h**) airlift bioreactor, (**i**) trickle bed or spray bioreactor, (**j**) mist bioreactor, (**k**) LCMB Bioreactor

steel isolating the roots from the impeller was fitted inside the culture vessel of stirred bioreactors. The cage or mesh acts as a matrix and supports the self-immobilization of the roots. This can improve the growth of hairy roots in stirred bioreactors and further bioreactor types as shown by Hilton and Rhodes (1990), Lee et al. (1999), Shadwick and Doran (2004), and also by studies with different hairy

root lines in our lab. In gas-phase bioreactors, there is an imperative need to immobilize the hairy roots since the continuous phase is gas. For these reasons, independent of the bioreactor type, immobilization of hairy roots by horizontal or vertical meshes as well as by cages or polyurethane foam meets the current standards (Wilson 1997). Reported productivities obtained from bioreactor cultivations of hairy roots are summarized in Table 8.5 and their schematics are depicted in Fig. 8.4. In addition to the previously mentioned modified stirred bioreactors, hairy roots were also cultivated in rotating and shaking representatives of mechanically driven bioreactors, different types of bubble column bioreactors as well as airlift bioreactors, the hydraulically driven radial flow bioreactor, and gas-phase bioreactors. Considering the bioreactor cultivation of hairy roots from an engineering standpoint, the most significant problem is the nonuniform distribution of biomass and, in particular, of high local root densities in the form of clumps. The latter is closely connected with mass transfer limitations and, in terms of efficient mass transfer (i.e., fluid mixing and oxygen transfer), presents considerable difficulties in bioreactor scaling-up. For laboratory scale, it is recognized that at hairy root concentrations up to 10 g dw L^{-1} the cultivation can be realized in essentially any bioreactor type listed in Table 8.5 (Curtis 2000).

Scale-up limitations due to high fluid flow resistance in dense root biomass at concentrations between 10 and 40 g dw L^{-1} were detected by Carvalho and Curtis (1998) in liquid-phase bioreactors. Yu et al. (1997) explained the growth-limiting effect of oxygen deficiency for hairy roots due to mass transport limitations under submerged cultivation conditions. Here gas–liquid oxygen transfer is hindered by reduced turbulence as a result of the roots present and also by coalescence of gas bubbles in the root clumps causing gas flow channeling around them. As a result, localized complete depletion of oxygen can arise. It has been suggested that liquid–solid mass transfer rather than gas–liquid mass transfer is the dominant influence on the rate of oxygen delivery to hairy roots. To improve the supply of oxygen to the growing roots in the bioreactor, different pneumatically driven bioreactor types have been developed such as the segmented bubble column operating with three spargers (Fig. 8.4e) and the convective flow bioreactor (Fig. 8.4f). Although the use of these bioreactors leads to higher biomass productivity for the configurations tested, scale-up problems can be expected (Carvalho and Curtis 1998; Kwok and Doran 1995).

The highest biomass productivity (>2 g dw L^{-1} d^{-1}) in the cultivation examples summarized in Table 8.5 was achieved by the radial flow bioreactor (Fig. 8.4b) and the BioWave (Fig. 8.4d). The radial flow bioreactor consists of a medium vessel (in which the culture medium is oxygenated externally), a peristaltic pump (which recirculates the aerated medium between the medium vessel and culture chamber), and a cylindrical culture chamber with an integrated wire mesh for root immobilization. After 12 days of cultivation, 51 g dw L^{-1} of beetroot hairy roots was harvested (Kino-Oka et al. 1999). In our lab, the application of the BioWave operating with Cellbag 2 L (total volume) at specific power input values between 30 and 50 Wm^{-3} yielded final dry biomass concentrations of 60 g dw L^{-1} d^{-1} for hyoscyamine (312 mg L^{-1}), which produces hairy roots of Egyptian henbane, and about 46.4 g dw L^{-1} d^{-1}

Table 8.5 Hairy root growth and metabolite production in suitable bioreactors

Product	Plant species	Bioreactor type	DCM_{final} (g dw L^{-1})	BMP_{dw} (g dw L^{-1} d^{-1})	Maximum metabolite productivity (mg L^{-1})	References
Hyoscyamine	*Datura stramonium*	Stirred bioreactor, Fig. 8.4a	8.4	0.24[#]	66.5	Hilton and Rhodes (1990)
Atropine	*Atropa belladonna*	Stirred bioreactor, Fig. 8.4a	60.2	1.81	325.1	Lee et al. (1999)
Antibody, murine IgG_1	*Nicotiana tabacum*	Stirred bioreactor, Fig. 8.4a	n.m.	n.m.	1.9	Shadwick and Doran (2004)
Atropine	*Atropa belladonna*	Bubble column, Fig. 8.4e	9.9	0.23	14	Kwok and Doran (1995)
Artemisin	*Artemisia annua*	Bubble column (*), feeding	15.3	0.4	0.025	Kim et al. (2002b)
Biomass	*Hyoscyamus muticus*	Bubble column, 8.4f	24.7	0.79	n.m.	Carvalho and Curtis (1998)
	Armoracia rusticana	Airlift bioreactor, Fig. 8.4h	11	0.35	n.m.	Taya et al. (1989b)
	Beta vulgaris	Radial flow bioreactor, Fig. 8.4b	51	3.73	n.m.	Kino-Oka et al. (1999)
	Hyoscyamus muticus	Rotated drum bioreactor, type Inversina, Fig. 8.4c	46	0.48	n.m.	Eibl et al. (1996)
	Hyoscyamus muticus	Plastic-lined bioreactor, Fig. 8.4g	8.8	0.28 (#)	n.m.	Curtis (2004)
	Hyoscyamus muticus	Spray bioreactor, Fig. 8.4i	31	1.1	n.m.	Not published
	Datura stramonium	Bubble column-spray bioreactor*	8 (#)	0.2	n.m.	Wilson (1997)
Hyoscyamine	*Hyoscyamus muticus*	BioWave, Fig. 8.4d, feeding	60	2.1	312	Eibl and Eibl (2006)
Ginsenosides	*Panax ginseng*	BioWave, Fig. 8.4d, feeding/exchange	46.4	0.51	145.6	Eibl and Eibl (2006)
Ginsenosides	*Panax ginseng*	Spray bioreactor Fig. 8.4i, exchange	9.9	0.17	39.2	Palazón et al. (2003)
L-DOPA	*Stizolobium hassjoo*	Mist bioreactor, Fig. 8.4j, feeding	11.5	0.72	644 (#)	Huang et al. (2004)

Nonexisting DCM_{final}: assumed to be 10% of FCM_{final}
n.m. – not measured
[#]BMP was calculated from DCM_{final} as a consequence of unavailable inoculum
*Wire-mesh cage or steel mesh

for ginsenosides (145.6 mg L^{-1}), which contain hairy roots of ginseng (Eibl and Eibl 2006). In this context, it is important to note that cultivations were carried out in feeding mode (200 mL → 500 mL), guaranteeing ebb-and-flow conditions for the growing hairy roots by changing the position of the BioWave rocker unit at constant rocking rate (6 rpm) and constant rocking angle (6°). The mat-like root tissue produced was uniformly distributed in the culture bag, which was completely filled with roots at the end of the cultivation. We are therefore of the opinion that the resulting reduction in mass transfer limitations caused biomass productivity to be ~40% higher than that achieved in the BioWave operating with culture bag 20 L (total volume). The root biomass was highly localized at three points in the culture bag 20 L. As far as root growth is concerned, a key to minimizing mass transfer limitations seems to be uniform distribution of root tissue in the bioreactor. Similar to BioWave, the Inversina (Fig. 8.4c), a rotating drum bioreactor characterized by oloid movement and low hydrodynamic shear, was run with alternate cycles of liquid- and gas phase, that is to say, in ebb-and-flow mode. When additional hairy root immobilization was guaranteed by meshes positioned at three levels in the Inversina bioreactor vessel, a final *Hyoscyamus muticus* dry biomass concentration of 46 g dw L^{-1} was obtained (Eibl et al. 1996).

Given that there are no limitations on the availability of oxygen to roots in a spray bioreactor (Fig. 8.4i), as described by Weathers et al. (1999), and that uniform biomass distribution is guaranteed, the question is why biomass productivity in a spray bioreactor does not exceed that of the BioWave for henbane and ginseng hairy roots. A possible explanation is that the lower biomass growth of both hairy root clones is caused by a nonoptimal spray cycle, which results in inadequate nutrient availability to the roots. It is clear that the availability of culture medium to the roots in spray and also mist bioreactors depends on the amount of droplets deposited on the root surface. This is a function of the spray or mist cycle (continuous/periodic/intermittent) as well as mist rate and the efficiency with which the hairy roots capture the droplets. We could not corroborate the hypothesis of Kim et al. (2002b) and Flores and Curtis (1992) that the importance of spray and mist bioreactors lies more in stimulating specific secondary metabolite production than in biomass production. In addition, the BioWave was superior to the spray reactor in ginsenoside production (Eibl and Eibl 2006).

Henbane and ginseng hairy roots grown in spray bioreactors possessed longer root hairs and were more abundant than those cultivated in the BioWave and the Inversina. These observations are consistent with the findings of Hofer as well as those of Williams and Doran. Doran also describes a much thicker layer of mucilage making mass transfer more difficult for hairy roots grown in liquid phases than for roots grown in gaseous phases (Williams and Doran 1999; Hofer 1996). However, it is also important to note that root hairs presenting a further significant barrier for mass transfer may affect the drainage of medium in gas-phase bioreactors (Shiao and Doran 2000). Ramakrishnan and Curtis (1994) demonstrated that profuse hairy roots entrain considerably more liquid than roots with fewer hairs. Because the number of hairs enhances spray/mist capture but may hinder liquid drainage in spray bioreactors, the mist bioreactor is preferable for the growth of profuse hairy roots (Kim et al. 2000a).

Uniform loading of hairy root matrices in the cultivation chamber and, most notably, achieving maximum use of the chamber are general problems for hairy root culture bioreactors. Whereas small-scale laboratory bioreactors are inoculated as open systems in a laminar flow, larger bioreactors necessitate the use of specific loading procedures and/or pharmaceutical isolator techniques for safe inoculation. For example, a pharmaceutical isolator is an integral part of the 50 L LCMB Bioreactor, a spray bioreactor of plastic film (Fig. 8.4k), which was introduced by Wink et al. (2005) for the production of podophyllotoxin. To allow hairy root attachment to the immobilization matrix, ebb-and-flow mode operation was included. The 500 L Wilson Bioreactor, which was specially designed for the investigation of hairy root growth in large scale, is a hybrid bioreactor (Table 8.5). It was used to cultivate hairy roots of *Datura stramonium* under submerged conditions for 21 days and subsequently in spray mode for 40 days. Besides the root immobilization assembly of wire chains and bars, it is specially constructed to allow mechanical root inoculation after growth of the root inoculum in a seed vessel. A total biomass of 39.8 kg fresh weight corresponding to 79.6 g fw L^{-1} was harvested (Wilson 1997). Although achieved biomass productivity was low, it was demonstrated that large-scale cultivation of hairy roots is realizable. In the same way as hairy root loading requires opening the bioreactor, so does the hairy root harvest after the process has finished. In the end, the hairy roots have to be detached from their immobilization matrices.

In summary, it can be stated that optimum bioreactor design for hairy root cultures and their products is a balance between, firstly, meeting the biological needs for efficient root tissue growth without inducing undesirable responses and/or supporting the secondary metabolite or recombinant protein expression in sufficient quantity and, secondly, quality.

8.3 Approaches to Improving Productivity in Plant Cell-Based Bioprocessing

Besides bioreactor type (Sect. 8.2), bioreactor operating mode is another appropriate strategy with which to pursue high biomass productivity and/or high bioactive compound (secondary metabolite, recombinant protein) content in plant cell-based production processes. As a matter of course, it is essential that a highly productive cell line is used, the inoculum density is sufficiently high, and that culture medium and cultivation parameters including pH, temperature, aeration rate, composition of the bioreactor's gas phase (O_2, CO_2, C_2H_4) and, if necessary, light duration and intensity are optimized (Sects. 8.1.1 and 8.1.2). To select the most suitable operating mode, the product formation pattern (growth association or nongrowth association and product secretion or intracellular accumulation) has to be taken into account. Furthermore, the strong dependence of this product formation pattern on the promoter used (constitutive or inducible) in recombinant protein production should be borne in mind (Shadwick and Doran 2004; Su 2006).

Productivity of growth-associated intra and extracellular products can be improved by increasing plant cell growth, prolonging the exponential growth phase, and increasing final biomass concentration in the bioreactor. Therefore, fed batch cultivations operating with intermittent feeding of sucrose or maltose are frequently used to enhance the productivity of biomass and intracellular products in plant cell cultivation processes (Choi et al. 2000; Huang et al. 2004; Wang et al. 2000). However, perfusion performed continuously at high perfusion rates (by cell retention devices such as spinfilters or by cell immobilization) yields considerably higher biomass concentrations and secondary metabolite or protein levels than batch or fed batch operations (Bentebibel et al. 2005). Indeed, this also depends on the efficient and easy removal of undesirable by-products and the resulting prolongation of active cell growth at high metabolic culture activity. However, it should be noted that, if product inhibition occurs, a product harvest unit (e.g., affinity column, adsorption or extraction column) may be coupled to the bioreactor to avoid cell growth rate reduction, cell decease, and product level lowering in growth-associated, extracellular products (Su 2006). For nongrowth associated, intracellular products, advantages can be found in two-stage batch cultivations in which growth phase and production phase have been decoupled by the application of a growth and production medium (Fujita et al. 1981; Raval et al. 2003; Zenk et al. 1977). In contrast, as a consequence of potential product inhibition, cultivations in perfusion mode should be chosen for nongrowth associated, extracellular products (Seki and Furusaki 1999).

It should be borne in mind that the most effective approach to significantly increasing the productivity of secondary metabolites is elicitation (Reuben and Croteau 2004). Elicitation (nontransgenic technique) has commercial potential and can result in metabolite secretion (Sevón 1997). It is defined as induction of gene expression associated with stimulation of secondary metabolite production by agents and means, the so-called elicitors. Elicitors are able to activate plant defense mechanisms, for example, synthesis of phytoalexins or active oxygen species such as H_2O_2 (known as the oxidative burst), in response to pathogenic attacks (Wu and Ge 2004). Biotic elicitors, which are microbe-derived molecules (polysaccharides, glycoproteins, bacterial and fungal cell walls, and low molecular weight compounds), and abiotic elicitors (ultraviolet radiation, ultrasonic pulsed electric fields, salts of heavy metals and various chemicals such as MeJA or JA) were successfully tested (Endress 1994; Lin et al. 2001; Lin and Wu 2002; Ye et al. 2004; Yeoman and Yeoman 1996). Results revealed that effective elicitation requires two-stage cultivation (because elicitors are often added to the culture medium after cell growth is complete), an optimum elicitor dosage and exposure time (Kim et al. 2002a; Rhijwani and Shanks 1998; Sevón 1997; Singh 1997). We would like to point out that in many cases the combination of enhancement techniques (multiple elicitors or combination of elicitation with immobilization, fed batch mode, perfusion mode, etc.) was advantageous not only for the quantity but also for the quality of the desired secondary metabolite (Bentebibel et al. 2005; Qian et al. 2005; Zhang et al. 2002).

Efforts have also been made to increase the accumulation and secretion of recombinant proteins in plant cell cultures by their retention (in situ adsorption) and especially their stabilization (with medium additives such as gelatin, bacitracin,

BSA, PVP, NaCl, DMSO, KNO_3, amino acids, or by osmolarity or pH shift) after protein has been produced (Shadwick and Doran 2004; Su 2006). However, further improvements in recombinant protein yields are necessary to increase the economic competitiveness of plant cell cultures when compared with their mammalian cell counterparts.

8.4 Application Examples and Potential Active Agent Candidates

Whereas maximum protein levels in plant cell cultures reach values of around 4% of total soluble protein (TSP) or 200 mg L^{-1} volumetric productivity (Hellwig et al. 2004), maximum volumetric productivity titers of recombinant proteins secreted by mammalian cells range from 500 to 5,000 mg L^{-1} (Wurm 2005). Nowadays, this means that between 2.5- and 25-fold higher protein levels can be guaranteed if mammalian cells, particularly CHO cells, are used as production organisms instead of plant cells. The first recombinant protein made by cultured tobacco suspension cells was human serum albumin (HSA) (Sijmons et al. 1990). To date, 20 different recombinant proteins have been successfully produced in proof-of-principle studies with plant cell cultures (Hellwig et al. 2004; Hülsing 2005; Kreis et al. 2001; Marshall 2006; Yazaki 2004; Vanisree et al. 2004; Voedisch et al. 2005). An updated summary of such PMPs (plant-made pharmaceuticals = protein-based medicines produced in transgenic plants and plant cells) is given in http://www.molecularfarming.com. In addition to cell lines derived from the tobacco cultivars Bright Yellow 2 (BY-2, doubling time of 13 h according to Nagata et al. 1992) and *Nicotiana tabacum* 1 (NT-1), well-characterized fast-growing transformed rice, alfalfa, tomato, soybean, and moss (*Physcomitrella*) cells are frequently applied to express important therapeutic proteins including antibodies, for example, AAT (for lung diseases), EPO (for anemia), GM-CSF (to stimulate bone marrow to produce white cells more quickly after chemotherapy), HBsAg (for hepatitis B prevention), and VEGF (for tumor diagnostics, and circulatory disorder) (Gorr and Wagner 2005; Ma et al. 2003; Schillberg et al. 2005). The first registration of such a PMP (vaccine against *Newcastle Disease Virus*) was announced from engineered plant cell cultures in February 2006 (Evans 2006). In order that recombinant proteins can also be manufactured by plant cell cultures in the future, cell banking of plant cells including their cryopreservation has to be established as both a master cell bank (MCB) and a working cell bank (WCB), which are prerequisites for GMP production.

While recombinant protein expression is still in its infancy, plant cell-based secondary metabolite production has a tradition of more than 45 years. Maximum secondary metabolite levels in plant cell cultures vary from 10 to 20% of the cell dry weight, which corresponds to productivities between 1 and 2 g L^{-1} d^{-1}. The plant cell-based product with the highest productivity (2 g L^{-1} d^{-1}) reported in the literature is rosmarinic acid (RA), a caffeic acid dimer described as antimicrobial, antiviral, and antioxidative (Parnham and Kesselring 1985). Since RA is most notably

used as a food additive and not as a high purity pharmaceutical, it is still commercially produced in the original way (i.e., by extraction from collected plant material), which is comparatively cheaper than the two-stage process with *Coleus blumei* suspension cells in stirred bioreactors (Kreis et al. 2001). It is worthy of note that the first industrial secondary metabolite production using *Lithospermum erythrorhizon* suspension cells was realized by Mitsui Petrochemical Industries, now Mitsui Chemicals, and concerned a naphtoquinone derivate called shikonin, a rich reddish-purple pigment for lipsticks that is also antimicrobial in activity. Under optimized conditions (two-stage mode), shikonin productivity averages out at $0.15\,g\,L^{-1}\,d^{-1}$ (Kreis et al. 2001). The shikonin production process is similar to ginsenoside (triterpenoid saponin) production (Nitto Denko Corporation), which has been routinely carried out in Japan since the 1980s. In the commercial ginsenoside production process, activities of 0.5 up to $0.7\,g\,L^{-1}\,d^{-1}$ were achieved in $25\,m^3$ stirred bioreactors operating with suspended *Panax ginseng* cells (Vanisree et al. 2004). We would like to point out that biotechnologically produced ginsenosides have been used as food additives (i.e., nutritional but not medical use) and whitening substances (cosmetics) since 1988 (Hibino and Ushiyama 1999). A landmark success in secondary metabolite manufacture was the industrial production of paclitaxel. Taxol is the generic name for the active ingredient of paclitaxel, an anti-cancer (ovarian, breast, and small-cell lung cancer) drug stopping cell division while stabilizing microtubule complexes (Wink et al. 2005). Cultivation of yew suspension cells in $70\,m^3$ stirred bioreactors (Phyton Biotech, Germany) ensured paclitaxel amounts exceeded those in intact plants, which constitute 0.01% of dry weight (Yazaki 2004). Further potential anticancer candidates being studied intensively in respect of their suitability for plant cell-based bioprocessing are camptothecin, podophyllotoxin, vincristine, vinblastine, and colchinine (Wink et al. 2005).

8.5 Conclusions

Plant cell-based bioprocessing aims to produce substances with antitumor, antiviral, hypoglycemic, anti-inflammatory, antiparasite, antimicrobial, tranquilizing, and immunmodulating activities, which can be categorized either as secondary metabolites or as recombinant proteins. Although to date only a few secondary metabolites and no PMPs have been manufactured commercially using plant cell cultures, their production in plant cell suspension cultures and hairy roots represents an alternative approach that may be advantageous when defined validated production conditions in compliance with GMP are required. When basic techniques are used to establish highly productive plant cell lines and successful cultivation in small-scale as well as large-scale bioreactors (m^3 range), basic techniques have been found to achieve moderate product yields for most target substances. An improved understanding of the manifold interactions between cultivated cells, product formation and bioreactor design, its features (fluid flow, mixing time, distribution time, oxygen transfer efficiency) affecting mass transfer and therefore product yield, will help not only to

enhance and sustain high productivity but also to reduce existing high process costs and exploit the potential of plant cell cultures up to large scale. By using scalable, disposable, and/or low cost bioreactors such as the BioWave, the WU bioreactor, or the SB bioreactor, a further reduction in production costs is possible. Close cooperation of molecular biologists, pharmacists, bioprocess development specialists as well as bioreactor and regulatory guidance specialists will contribute to innovative new solutions. Plant cell-based bioprocessing can therefore be expected to increase in importance in the future.

8.6 Questions and Problems

1. Discuss the main operational parameters for optimal plant cell growth on the basis of plant cell characteristics.
2. Specify the constituents of plant cell culture media and describe their functions in mass propagation, cell differentiation, and metabolite production.
3. Compare the establishment, maintenance, and in vitro growth as well as secondary metabolite and recombinant protein production of callus cultures, plant cell suspension cultures, and hairy root cultures.
4. Explicate and review common methods of cell growth determination in plant cell-based processes and compare them with those of animal cell-based production processes.
5. You have the task of determining growth kinetics, maximum BMP, and doubling time of grape suspension cells growing in 250-mL shake flasks (25 °C, 100 rpm, darkness, batch) over 21 days. Per shake flask 50-mL modified MS medium has to be inoculated with 2.5 g fw of exponentially growing grape cell biomass with viability over 90%. Plan the experiment and discuss the experimental set up and analysis methods.
6. Describe possible signs of shear damage in plant cells cultivated in bioreactors.
7. Give reasons for the predominance of stirred bioreactors for plant cell suspension cultivation at high cell densities.
8. A genetically engineered BY-2 tobacco cell line has been used to produce hemoglobin in BioWave 20 SPS (CultiBag 20 L). Growth in batch culture has been monitored and the results summarized in Table 8.6. Determine maximum BMP and culture doubling time. Assess the growth behavior and discuss relevant approaches to improving it.
9. Hairy roots of *Harpagophytum procumbens* producing harpagosides grow in Petri dishes with doubling times of 7 days. At 25 °C and darkness, the roots are characterized by long hairs and intense hairiness on modified solid B5 medium. Develop and elucidate scale-up procedures for the application of suitable laboratory bioreactors. Decide which bioreactor type is theoretically most suitable for harpagoside expression that has been detected to have occurred due to growth association. In addition, discuss where and under what conditions you would expect mass transfer limitations, which result in low growth and harpagoside production.

Table 8.6 Experimental data

Time (d)	Biomass dry weight (g dw L^{-1})
0	0.02
3	0.05
5	1.47
7	10.2
9	12.1
11	11.9
13	9.6
15	9.55
17	8.35
19	7.2

10. Explain the most important strategies used for the enhancement of secondary metabolite production in plant cell cultures for growth- and nongrowth-associated processes.
11. Give examples of secondary metabolites and recombinant proteins produced by plant cell cultures, and describe their characteristics. What conditions are required for a high productivity process based on plant cell cultures?

List of Abbreviations and Symbols

AAT	alpha-1-antitrypsin
ABA	abscisic acid
Ala	alanine
Arg	arginine
Asn	asparagine
B	boron
BAP	6-benzylaminopurine
BMP	biomass productivity
BSA	bovine serum albumin
BY-2	Bright Yellow 2
CER	carbon dioxide evolution rate
Co	cobalt
CO_2	carbon dioxide
Cu	copper
Cys	cysteine
C_2H_4	ethylene
DCM	dry cell mass (dry biomass concentration)
DMSO	dimethyl sulphoxide
DS	dry substance
d	day
dw	dry weight

EDTA	ethylenediamine tetraacetic acid
EPO	erythropoietin
FCM	fresh cell mass (fresh biomass concentration)
cGMP	current Good Manufacturing Practice
f	final
fw	fresh weight
g	gram
GA_3	gibberellin A_3
GA_4	gibberellin A_4
GA_7	gibberellin A_7
GI	growth index
GM-CSF	granulocyte macrophage colony stimulating factor
His	histidine
h	hour
HBsAg	Hepatitis B surface antigen
HSA	human serum albumin
H_2O_2	hydrogen peroxide
I	iodine
i	initial
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
Ile	isoleucine
IPC	In-Process control
JA	jasmonic acid
kDa	kilodalton
k_La	oxygen transfer coefficient
klux	kilolux
KNO_3	potassium nitrate
L	Liter
L-DOPA	3,4-dihydroxyphenylalanine
Leu	leucine
MCB	master cell bank
MeJA	methyl jasmonate
Met	methionine
mg	milligram
min	minute
mm	millimeter
Mn	manganese
Mo	molybdenum
MS	medium Murashige and Skoog medium
NAA	naphthaleneacetic acid
NaCl	sodium chloride
NT-1	Nicotiana tabacum 1
OUR	oxygen uptake rate

O_2	oxygen
pCV	packed cell volume
pCO_2	part of dissolved carbon dioxide in the medium
pO_2	part of dissolved oxygen in the medium
PEG	polyethyleneglycol
PMP	plant-made pharmaceutical
PVP	polyvinylpyrrolodine
PVS2	plant vitrification solution 2
r	recombinant
RA	rosmarinic acid
Ri	root-inducing
rpm	revolution per minute
T-DNA	transfer DNA
t	cultivation time
TSP	total soluble protein
tx	point x in time
V	volume
VEGF	vascular endothelial growth factor
vir	virulence
vvm	volumes per volume per minute
W	watt
WCB	working cell bank
Zn	zinc
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
μm	micrometer
μmole	micromole
°C	degree Centrigrade

Acknowledgement We wish to thank Dr. Kirsi-Marja Oksman-Caldentey for critical reading of the manuscript and her helpful comments.

References

Abdullah M, Ariff A, Marziah M, Ali A, Lajis N (2000) Strategies to overcome foaming and wall growth during the cultivation of *Morinda elliptica* cell suspension culture in a stirred-tank bioreactor. Plant Cell Organ Cult 60:205–212

Bais HP, Suresh B, Raghavarao KSMS, Ravishankar GA (2002) Performance of hairy root cultures of *Cichorium intybus L.* in bioreactors of different configurations. In Vitro Plant 38:573–580

Bates TR, Lynch JP (1996) Stimulation of hairy root elongation in *Arabidopsis thaliana* by low phosphorous availability. Plant Cell Environ 19:529–538

Benson EE, Lynch PT, Stacey GN (1998) Advances in plant cryopreservation technology: Current applications in crop plant biotechnology. AgBiotech News Inf 5:133N–142N

Bentebibel S (2003) Estudio de la produccion de taxanos por cultivos de células en suspensio e immovilizadas de *Taxus baccata*. PhD Thesis, University of Barcelona

Bentebibel S, Moyano E, Palazón J, Cusidó RM, Bonfill M, Eibl R, Pinol MT (2005) Effects of immobilization by entrapment in alginate and scale-up on paclitaxel and baccatin III production in cell suspension cultures of *Taxus baccata*. Biotechnol Bioeng 89:647–655

Bergmann L (1967) Wachstum grüner Suspensionskulturen von *Nicotiana tabacum* var. "Samsun" mit CO_2 als Kohlenstoffquelle. Planta 74:243–249

Bhojwani SS, Razdan MK (1983) Developments in Group Science 5. Elsevier, Amsterdam

Bidney D, Scelonge C, Martich J, Burrus M, Sims L, Huffmann G (1992) Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Mol Biol 18:301–313

Bordonaro JL, Curtis WR (2000) Inhibitory role of root hairs on transport within root culture bioreactors. Biotechnol Bioeng 70:176–86

Calderón AA, Zapata JM, Ros Barceló A (1994) Differential expression of a cell wall-localized peroxidase isoenzyme capable of oxidizing 4-hydroxystilbenes during the cell culture of grapevine (*Vitis vinifera cv*. Airen and Monastrell). Plant Cell Tissue Organ Cult 37:121–127

Carvalho EB, Holihan S, Pearsall B, Curtis WR (1997) Effect of root morphology on reactor design and operation for production of chemicals. In: Doran PM (ed) Hairy Roots: Culture and Application. Harwood Academic, Amsterdam, pp 151–167

Carvalho EB, Curtis WR (1998) Characterization of fluid-flow resistance in root cultures with a convective flow tubular bioreactor. Biotechnol Bioeng 60:375–384

Chattopadhyay S, Mehra RS, Srivastava AK, Bhojwani SS, Bisaria VS (2003) Effect of major nutrients on podophyllotoxin production in *Podophyllum hexandrum* suspension cultures. Appl Microbiol Biotechnol 60:541–546

Chattopadhyay S, Farkya S, Srivastava AK, Bisaria VS (2002) Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. Biotechnol Bioproc Eng 7:138–149

Chilton MD, Tepfer D, Petit A, David C, Casse-Delbart F, Tempe J (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of host plant cells. Nature 295:432–434

Choi HK, Kim SI, Son JS, Hong SS, Lee HS, Chung IS, Lee HJ (2000) Intermittent maltose feeding enhances paclitaxel production in suspension culture of *Taxus chinensis* cells. Biotechnol Lett 22:1793–1796

Cuperus S, Rouvinez V, Eibl R, Kneubühl M, Müller J, Hühn T, Amadó R (2006) Potential of the BioWave reactor for plant cell-based bioprocesses: *V. vinifera* cell suspension culture and their metabolites as a case study. In: Sorvari S, Toldi O (eds) Proceedings for Second International Conference on Bioreactor Technology in Cell, Tissue Culture and Biomedical Applications, Saariselkä, Lapland, 27–31 March 2006

Curtis WR (2004) Growing cells in a reservoir formed of a flexible sterile plastic liner. United States Patent, 6,709,862 B2

Curtis WR, Emery A (1993) Plant cell suspension culture rheology. Biotechnol Bioeng 42:520–526

Curtis WR (2000) Hairy roots, bioreactor growth. In: Spier RE (ed). Encyclopedia of Cell Technology, vol 2. Wiley VCH, New York, pp 827–841

Davioud E, Kan C, Hamon J, Tempé J, Husson HP (1989) Production of indole alkaloids by in vitro root cultures from *Catharanthus trichophyllus*. Phytochem 28:2675–2680

Decker EL, Reski R (2004) The moss bioreactor. Curr Opin Plant Biol 7:166–170

Deus-Neumann B, Zenk HM (1984) Instability of indole alkaloid production in *Catharanthus roseus* cells in suspension cultures. Planta Med 50:427–431

DiIorio AA, Cheetham RD, Weathers PJ (1992) Carbon dioxide improves the growth of hairy roots cultured on solid medium and in nutrient mists. Appl Microbiol Biotechnol 37:463–467

Do CB, Cormier F (1991) Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (*Vitis vinifera*) cell suspensions. Plant Cell Rep 9:500–504

Doran PM (2002) Properties and applications of hairy root cultures. In: Oksman-Caldentey KM, Barz WH (eds) Plant Biotechnology and Transgenic Plants. Marcel Dekker, New York, pp 143–161

Doran PM (1993) Design of reactors for plant cells and organs. Adv Biochem Eng 48:115–168

Dubuis D (1994) Design and scale-up of bubble-free fluidised bed reactors for plant cell cultures. PhD Thesis, Swiss Federal Institute of Technology, Zurich

Ducos JP, Lambot C, Pètiard V (2007) Bioreactors for coffee mass propagation by somatic embryogenesis. Int J Dev Biol 1:1–12

Eibl R, Eibl D (2002) Bioreactors for plant cell and tissue cultures. In: Oksman-Caldentey KM, Barz WH (eds) Plant Biotechnology and Transgenic Plants. Marcel Dekker, New York, pp 163–199

Eibl R, Hans D, Warlies S, Lettenbauer C, Eibl D (1999) Einsatz eines Taumelreaktorsystems mit interner Beleuchtung. BioWorld 2:10–12

Eibl R, Eibl D (2006) Design and use of the Wave Bioreactor for plant cell culture. In: Dutta Gupta S, Ibaraki Y (eds) Plant Tissue Culture Engineering, Series: Focus on Biotechnology, vol 6. Springer, Dordrecht, pp 203–227

Eibl R, Eibl D (2008) Design of bioreactors suitable for plant cell and tissue cultures. Phytochem Rev, DOI 10.1007/s11101-007-9083-z

Eibl R, Hans D, Eibl D (1996) Vergleichende Untersuchungen zur Kultivierung pflanzlicher Zellen in Laborbioreaktoren. In: iba (ed) Proceedings zum 8. Heiligenstädter Kolloquium "Technische Systeme für Biotechnologie und Umwelt", Heiligenstadt, Germany, pp 100–105

Endress R (1994) Plant Cell Biotechnology. Springer, Berlin, Heidelberg New York

Evans DA, Sharp WR, Ammirato PV, Yamada Y (1983) Handbook of Plant Cell Culture, vol 1. Macmillan, New York

Evans D, Coleman J, Kearns A (2003) Plant Cell Culture. BIOS Scientific, Abingdon

Evans J (2006) Plant-Derived Drug. http://www.rsc.org/chemistryworld/News/2006/February/07020602.asp. Cited 10 Apr 2007

Fischer U, Santore UW, Hüsemann W, Barz W, Alfermann AW (1995) Semicontinuous cultivation of photoautotrophic cell suspension cultures in a 20 L airlift-reactor. Plant Cell Tissue Organ Cult 38:123–134

Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM (2004) Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 7:152–158

Flores HE (1987) Use of plant cells and organ culture in the production of biological chemicals. In: LeBaron HM, Mumma RO, Honeycutt RC, Duesing JH (eds) Biotechnology in Agricultural Chemistry. ACS Symp Ser 334, Washington, pp 66–86

Flores HE, Curtis WR (1992) Approaches to understanding and manipulating the biosynthetic potential of plant roots. Ann NY Acad Sci 665:188–209

Fowler MW (1988) Problems in commercial exploitation of plant cell cultures. In: Bock B, Marsh J (eds) Application of Plant Cell and Tissue Culture. Ciba Foundation Symposium 137. Wiley, Chichester, pp 239–253

Fujita H, Hara Y, Suga C, Morimoto T (1981) Production of shikonin derivates by cell suspension cultures of *Lithospermum erythrorhizon* II: A new medium for the production of shikonin derivates. Plant Cell Rep 1:61–63

Gamborg OL, Miller RA, Ojimak K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Georgiev M, Pavlov A, Bley T (2006) Betalains by transformed *Beta vulgaris* roots in stirred tank bioreactor: batch and fed-batch process. In: FinMed (ed) Proceedings for Second International Conference on Bioreactor Technology in Cell, Tissue Culture and Biomedical Applications, Saariselkä, Lapland, p 35

Girard LS, Fabis MJ, Bastin M, Courtois D, Pétiard V, Koprowski H (2006) Expression of a human anti-rabies virus monoclonal antibody in tobacco cell culture. BBRC 345:602–607

Glicklis R, Mills D, Sitton D, Stortelder W, Merchuk JC (1998) Polysaccharide production by plant cells in suspension: Experiments and mathematical modelling. Biotechnol Bioeng 57:732–740

Gorr G, Wagner S (2005) Humanized glycosylation: Production of biopharmaceuticals in a moss bioreactor. In: Knäblein J (ed) Modern Biopharmaceuticals, vol 3. Wiley VCH, Weinheim, pp 919–929

Guardiola J, Iborra JL, Cánovas M (1994) A model that links growth and secondary metabolite production in plant cell suspension cultures. Biotechnol Bioeng 46:291–297

Hall R, Yeoman M (1987) Intercellular and intercultural heterogeneity in secondary metabolite accumulation in cultures of *Catharanthus roseus* following cell line selection. J Exp Bot 38:1391–1398

Hamill JD, Lidgett AJ (1997) Hairy root cultures: Opportunities and key protocols for studies in metabolic engineering. In: Doran PM (ed) Hairy Roots: Culture and Applications. Harwood, Amsterdam, pp 1–29

Hamill JD, Parr AJ, Robins RJ, Rhodes MJC (1986) Secondary product formation by cultures of *Beta vulgaris* and *Nicotiana rustica* transformed with *Agrobacterium rhizogenes*. Plant Cell Rep 5:111–114

Han B, Linden JC, Gujarathi NP, Wickramasinghe SR (2004) Population balance approach to modelling hairy root growth. Biotechnol Prog 20:872–879

Hansen G, Chilton MD (1996) "Agrolistic" transformation of plant cells: Integration of T-strands generated in planta. Proc Natl Acad SCI USA 94:7469–7474

Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nature Biotech 22:1415–1422

Hess D (1992) Biotechnologie der Pflanzen. Eugen Ulmer, Stuttgart

Hibino K, Ushiyama K (1999) Commercial production of ginseng by plant tissue culture technology. In: Fu TJ, Curtis WR (eds) Plant Cell and Tissue Culture for the Production of Food Ingredients. Kluwer, New York, pp 215–224

Hilton MG, Wilson PDG (1995) Growth and uptake of sucrose and mineral ions by transformed root cultures of *Datura stramonium, Datura candida x aurea, Datura wrightii, Hyoscyamus muticus* and *Atropa belladonna*. Planta Med 61:345–350

Hilton MG, Rhodes MJC (1990) Growth and hyoscyamine production of hairy root cultures of *Datura stramonium* in a modified stirred tank reactor. Appl Microbiol Biotechnol 33:132–138

Hjortso MA (1997) Mathematical modelling of hairy root growth. In: Doran PM (ed) Hairy Roots: Culture and Applications. Harwood, Amsterdam, pp 169–178

Hofer R (1996) Root hairs. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant Roots: The Hidden Half. Marcel Dekker, New York, pp 111–126

Huang SY, Hung CH, Chou SN (2004) Innovative strategies for operation of mist trickling reactors for enhanced hairy root proliferation and secondary metabolite productivity. Enzyme Microb Technol 35:22–32

Hu ZB, Du M (2006) Hairy root and its application in plant genetic engineering. J Integr Plant Biol 48:121–127

Hülsing B (2005) Biopharmazeutika aus transgenen Pflanzen. BioWorld 3:10–12

James E, Lee JM (2001) The production of foreign proteins from genetically modified plant cells. In: Tscheper T (ed) Advances in biochemical engineering: Plant cells. vol 72. Springer, Berlin Heidelberg, pp 127–156

Joliceur M, Chavarie C, Carreau PJ, Archambault J (1992) Development of a helical-ribbon impeller bioreactor for high-density plant cell suspension culture. Biotechnol Bioeng 39:511–521

Kartha KK (1987) Advances in the cryopreservation technology of plant cells and organs. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) Plant Tissue and Cell Culture. Plant Biology 3. Liss, New York, pp 447–458

Kawase Y, Moo-Young M (1990) The effect of antifoam agents on mass transfer in bioreactors. BioProcess Eng 5:169–173

Kieran PM, MacLoughlin PF, Malone DM (1997) Plant cell suspension cultures: Some engineering considerations. J Biotechnol 59:39–52

Kieran PM, Malone DM, MacLoughlin PF (2000) Effects of hydrodynamic and interfacial forces on plant cell suspension systems. In: Tscheper T, Schügerl K, Kretzmer G (eds) Advances in Biochemical Engineering Biotechnology: Influence of Stress on Cell Growth and Product Formation, vol 67. Springer, Berlin Heidelberg New York, pp 139–177

Kim DI, Cho GH, Pedersen H, Chin CK (1991) A hybrid reactor for high density cultivation of plant cells. Appl Microbiol Biotechnol 34:726–729

Kim S, Hopper E, Hjortso MA (1995) Hairy root growth models: Effect of different branching patterns. Biotechnol Prog 11:178–186
Kim Y, Wyslouzil BE, Weathers PJ (2002a) Secondary metabolism of hairy root cultures in bioreactors. In Vitro Cell Dev Biol Plant 38:1–10
Kim Y, Wyslouzil BE, Weather PJ (2002b) Growth of *Artemisia annua* hairy roots in liquid- and gas-phase reactors. Biotechnol Bioeng 80:454–464
Kino-Oka M, Hitaka Y, Taya M, Tone S (1999) High-density culture of red beet hairy roots by considering medium flow conditions in a bioreactor. Chem Eng Sci 54:3179–3186
Kobayashi T, Niino T, Kobayashi M (2006) Cryopreservation of tobacco BY-2 suspension cell cultures by vitrification with encapsulation. Plant Biotech 23:333–337
Komari T, Ishida Y, Hiei Y (2004) Plant transformation technology: Agrobacterium-mediated transformation. In: Christou P, Klee H (eds) Handbook of Plant Biotechnology, vol 1. Wiley, Chichester, pp 233–261
Kreis W, Baron D, Stoll G (2001) Biotechnologie der Arzneistoffe. Deutscher Apotheker Verlag, Stuttgart
Krüger A (2006) Untersuchungen zur in vitro Kultivierung von Zell- und Gewebekulturen des *Malus domestica*. Diploma Thesis, University of Applied Sciences Köthen/Anhalt
Kwok KH, Doran PM (1995) Kinetic and stoichiometric analysis of hairy roots in a segmented bubble column reactor. Biotechnol Prog 11:429–435
Lee KT, Suzuki T, Yamakawa T, Kodama T, Igarashi Y, Shimomura K (1999) Production of tropane alkaloids by transformed root cultures of *Atropa belladonna* in stirred bioreactors with stainless steel net. Plant Cell Rep 18:567–571
Lin LD, Wu JY, Ho KP, Qi SY (2001) Ultrasonic-induced physiological effects and secondary metabolite (saponin) production in *Panax ginseng* cell cultures. Ultrasound Med Biol 27:1147–1152
Lin LD, Wu JY (2002) Enhancement of shikonin production in single- and two-phase suspension cultures of *Lithospermum erythrorhizon* cells using low-energy ultrasound. Biotechnol Bioeng 78:81–88
Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805
Madhusudhan R Ramachandra Rao S, Ravishankar GA (1995) Osmolarity as a measure of growth of plant cells in suspension cultures. Enzyme Microb Technol 17:989–991
Mantell SH, Smith H (1983) Plant Biotechnology. Cambridge University Press, Cambridge
Marshall B (2006) MolecularFarming.com. http://www.molecularfarming.com/PMPs-and-PMIPs.html. Cited 10 Apr 2007
Menges M, Murray JAH (2004) Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures. Plant J 37:635–644
Mirjalili N, Linden JC (1995) Gas phase composition effects on suspension cultures of *Taxus cuspidata*. Biotechnol Bioeng 48:123–132
Misawa M (1994) Plant tissue culture: An alternative for production of useful metabolites. FAO Agricultural Services Bulletin N 108. Food and Agriculture Organization of the United Nations, Rome, p 95
Moldenhauer JR (2003) Cell culture preservation and storage for industrial bioprocesses. In: Vinci VA, Parekh SR (eds) Handbook of Industrial Cell Culture: Mammalian, Microbial and Plant Cells. Humana Press, Totowa, New Jersey, pp 498–501
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479
Murashige T (1973) Nutrition of plant cells and organs in vitro. In Vitro 9:81–85
Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. Int Rev Cytol 132:1–30
Naill MC and Roberts SC (2005) Cell cycle analysis of *Taxus* suspension cultures at the single cell level as an indicator of culture heterogeneity. Biotechnol Bioeng 90:491–500
Nettleship L, Slaytor M (1974) Adaption of *Peganum harmala* callus to alkaloid production. J Exp Bot 25:1114–1123

Nuutila AM, Toivonen L, Kauppinen V (1994) Bioreactor studies on hairy root cultures of *Catharanthus roseus*: Comparison of three bioreactor types. Biotechnol Techniques 8:61–66

Nuutila AM, Linquist AS, Kauppinen V (1997) Growth of hairy root cultures of strawberry (*Fragaria ¥ ananassa Duch*.) in three different types of bioreactors. Biotechnol Techniques 6:363–366

Oksman-Caldentey KM, Sevón KM, Vanhala L, Hiltunen R (1994) Effect of nitrogen and sucrose on the primary and secondary metabolism of transformed root cultures of *Hyoscyamus muticus*. Plant Cell Tissue Org Cult 38:263–272

Oksman-Caldentey KM, Hiltunen R (1996) Transgenic crops for improved pharmaceutical products. Field Crops Res 45:57–69

Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tiss Org Cult 81:287–300

Palazón J, Mallol A, Eibl R, Lettenbauer C, Cusidó RM, Piñol MT (2003) Growth and ginsenoside production in hairy root cultures of *Panax ginseng* using a novel bioreactor. Planta Med 69:344–349

Pan ZW, Wang HQ, Zhong JJ (2000) Scale-up study on suspension cultures of *Taxus chinensis* cells for production of taxane diterpene. Enzyme Microb Tech 27:714–723

Parnham MJ, Kesselring K (1985) Rosmarinic acid. Drugs Future 10:756–757

Petersen M, Dombrowski K, Gertlowski C, Haeusler E, Kawatzki B, Meinhard J, Alfermann AW (1992) The use of plant cell cultures to study natural product biosynthesis. In: Oono K, Hirabayashi T, Kikuchi S, Handa H, Kajiwara K (eds) Plant Tissue Culture and Gene Manipulation for Breeding and Formation of Phytochemicals. NAIR, Tsukuba, pp 293–296

Präve P, Faust U, Sittig W, Sukatsch DA (1994) Handbuch der Biotechnologie. Oldenbourg Verlag, München, p 189

Qian ZG, Zhao ZJ, Xu Y, Qian X, Zhong JJ (2005) Highly efficient strategy for enhancing taxoid production by repeated elicitation with a newly synthesized jasmonate in fed-batch cultivation of *Taxus chinensis* cells. Biotechnol Bioeng 90:516–521

Ramakrishnan D, Curtis WR (1994) Fluid dynamic studies on plant root cultures for application to bioreactor design. In: Ryu DD, Furusaki S (eds) Studies in Plant Science, 4. Advances in Plant Biotechnology: Production of Secondary Metabolites. Elsevier, Amsterdam, pp 281–305

Rasmussen JL, Kikkert JR, Roy MK, Sanford JC (1994) Biolistic transformation of tobacco and maize suspension cells using bacterial cells as microprojectiles. Plant Cell Rep 13:212–217

Raval KN, Hellwig S, Prakash G, Ramos-Plascencia A, Srivastava A, Büchs J (2003) Necessity of two-stage process for the production of Azadirachtin-related limonoids in suspension cultures of *Azadirachata indica*. J Biosc Bioeng 96:16–22

Reinert J, Yeoman MM (1982) Plant Cell and Tissue Culture: A Laboratory Manual. Springer, Berlin Heidelberg New York

Reuben JP, Croteau RB (2004) Metabolic Engineering of plant secondary metabolism. In: Christou P, Klee H (eds) Handbook of Plant Biotechnology, vol 1. Wiley, New York, pp 609–627

Rhijwani SK, Shanks JV (1998) Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14:442–449

Rhodes MJC, Parr AJ, Giulietti A, Aird ELH (1994) Influence of exogeneous hormones on the growth and secondary metabolite formation in transformed root cultures. Plant Cell Tissue Organ Cult 38:143–151

Roberts SG, Shuler ML (1997) Large-scale plant cell culture. Curr Opin Biotech 8:154–159

Ryu DDY, Lee SO (1990) Determination of growth rate for plant cell cultures: Comparative studies. Biotechnol Bioeng 35:305–311

Saito K, Mizukami H (2002) Plant cell cultures as producers of secondary metabolites. In: Oksman-Caldentey KM, Barz WH (eds) Plant Biotechnology and Transgenic Plants. Marcel Dekker, New York, pp 77–109

Schiermeyer A, Dorfmüller S, Schinkel H (2004) Production of pharmaceutical proteins in plants and plant cell suspension cultures. In: Fischer R, Schillberg S (eds) Molecular Farming. Wiley-VCH, Weinheim, pp 91–112

Schillberg S, Twyman RM (2004) Emerging production systems for antibodies in plants. In: Christou P, Klee H (eds) Handbook of Plant Biotechnology, vol 2. Wiley, Chichester, pp 801–810

Schillberg S, Twyman RM, Fischer R (2005) Biopharmaceutical production in cultured plant cells. In: Knäblein J (ed) Modern biopharmaceuticals, vol 3. Wiley VCH, Weinheim, pp 949–965

Schumacher HM, Malik KA, van Iren F (1994) Simple storage of plant cell cultures in liquid media. Publication No. 13, UNESCO/WFCC Technical Information Sheet:1–4

Seki M, Furusaki S (1999) Medium recycling as an operational strategy to increase plant secondary metabolite formation. In: Fu TJ, Curtis WR (eds) Plant cell and tissue culture for the production of food ingredients. Kluwer, New York, pp 157–163

Seitz U (1987) Cryopreservation of plant cell cultures. Planta Med 53:311–314

Sevón N (1997) Tropan alkaloids in hairy roots and regenerated plants of *Hyoscyamus muticus*. PhD Thesis, University of Helsinki

Sevón N, Oksman-Caldentey KM (2002) *Agrobacterium rhizogenes*-mediated transformation: Root cultures as a source of alkaloids. Planta Med 68:859–868

Shadwick FS, Doran PM (2004) Foreign protein expression using plant cell suspension and hairy root cultures. In: Fischer R, Schillberg S (eds) Molecular Farming. Wiley-VCH, Weinheim, pp 13–36

Sharp JM, Doran PM (2001) Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. Biotechnol Prog 17:979–992

Shiao TL, Doran PM (2000) Root hairiness: Effect on fluid flow and oxygen transfer in hairy root cultures. J Biotechnol 83:199–210

Shibasaki N, Hirose K, Yonemoto T, Takadi T (1992) Suspension culture of *Nicotiana tabacum* cells in a rotary-drum bioreactor. J Chem Technol Biotechnol 53:359–363

Sijmons PC, Dekker BMM, Schrammeijer B, Verwoerd TC, van den Elzen PJM, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. Bio/Technology 8:217–221.

Singh G, Curtis WR (1994) Reactor design for plant root culture. In: Shargool PD, Ngo TT (eds) Biotechnological Applications Plant Cultures. CRC Press. Boca Raton, FL, pp 185–206

Singh G (1997) Elicitation-manipulating and enhancing secondary metabolite production. In: Fu TJ, Curtis WR (eds) Plant cell and tissue culture for the production of food ingredients. Kluwer, New York, pp 101–127

Steward N, Martin R, Engasser JM, Goergen JL (1999) Determination of growth and lysis kinetics in plant cell suspension cultures from the measurement of esterase release. Biotechnol Bioeng 66:114–121

Storhas W (1994) Bioreaktoren und periphere Einrichtungen. Vieweg, Braunschweig/Wiesbaden

Su WW (2006) Bioreactor engineering for recombinant protein production using plant cell suspension culture. In: Dutta Gupta S, Ibaraki Y (eds) Plant Tissue Culture Engineering, Series: Focus on Biotechnology, vol 6. Springer, Dordrecht, pp 135–159

Su WW (1995) In situ filtration of *Anchusa officinalis* culture in a cell-retention stirred tank bioreactor. Biotechnol Techniques 9:259–264

Takahashis S, Fujita Y (1991) Production of shikonin. In: Komamine A, Misawa M, DiCosmo F (eds) Plant Cell Culture in Japan. CMC, Tokyo, pp 72–78

Takayama S, Akita M (2006) Bioengineering aspects of bioreactor application in plant propagation. In: Dutta Gupta S, Ibaraki Y (eds) Plant Tissue Culture Engineering, Series: Focus on Biotechnology, vol 6. Springer, Dordrecht, pp 83–100

Takebe J, Labib G, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwiss 5:318–320

Tanaka H, Uemura M, Kaneko Y, Aoyagi H (1993) Estimation of cell biomass in plant cell suspensions by the osmotic pressure measurement of culture broth. J Ferment Bioeng 76:501–504

Tanaka H (2000) Technological problems in cultivation of plant cells at high density. Biotechnol Bioeng 67:1203–1217

Tanaka H, Nishijima F, Suwa M, Iwamoto T (1983) Rotating drum fermentor for plant cell suspension cultures. Biotechnol Bioeng 25:2359–2370

Taticek RA, Lee CWT, Shuler ML (1994) Large-scale insect and plant cell culture. Curr Opin Biotechnol 5:165–174

Taya M, Hegglin M, Prenosil JE, Bourne JR (1989a) On-line monitoring of cell growth in plant tissue cultures by conductometry. Enzyme Microb Technol 11:170–176

Taya M, Yoyoma A, Kondo O, Kobayashi T, Matsui C (1989b) Growth characteristics of plant hairy roots and their cultures in bioreactors. J Chem Eng Jpn 22:84–89

Taya M, Kino-Oka M, Tone S (1989c) A kinetic model of branching growth of plant hairy root. J Chem Eng Jpn 22:698–670

Terrier B, Courtois D, Hénault N, Cuvier A, Bastin M, Aknin A, Dubreuil J, Pétiard V (2006) Two new disposable bioreactors for plant cell culture: The wave & undertow bioreactor and the slug bubble bioreactor. Biotechnol Bioeng, Early View: 1–25

Than NT, Murthy HN, Yu KW, Jeong CS, Hahn EJ, Paek KY (2004) Effect of inoculum size on biomass accumulation and ginsenoside production by large-scale cell suspension cultures of *Panax ginseng*. J Plant Biotechnol 6:265–268

Towler MJ, Kim Y, Wyslouzil BE, Correll MJ, Weathers PJ (2006) Design, development and applications of mist bioreactors for micropropagation and hairy root culture. In: Dutta Gupta S, Ibaraki Y (eds) Plant Tissue Culture Engineering, Series: Focus on Biotechnology, vol 6. Springer, Dordrecht, pp 119–134

Twyman RM, Christou P (2004) Plant transformation technology: Particle bombardment. In: Christou P, Klee H (eds) Handbook of Plant Biotechnology, vol 1. Wiley, Chichester, pp 263–289

Val J (1993) Modelling the physiology of plant cells in suspension culture. PhD Thesis, Leiden University

Valluri JV, Treat WJ, Soltes E (1991) Bioreactor culture of heterotrophic sandalwood (*Santalum album* L.) cell suspensions utilizing a cell-lift impeller. Plant Cell Rep 10:366–370

Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY, Tsay HS (2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot Bull Acad Sin 45:1–22

Vervliet G, Holsters M, Teuchy H, van Montagu M, Schell J (1975) Characterization of different plaqueforming and defective temperature phages in Agrobacterium strains. J Gen Virol 26:33–48

Voedisch B, Menzel C, Jordan E, El-Ghezal A, Schirrmann T, Hust M, Jostock T (2005) Heterologe Expression von rekombinanten Proteinpharmazeutika. Laborwelt 6:26–31

Wang HQ, Yu JT, Zhong JJ (2000) Significant improvement of taxane production in suspension cultures of *Taxus chinensis* by sucrose feeding strategy. Process Biochem 35:479–483

Wang SJ, Zhong JJ (1996a) A novel centrifugal impeller bioreactor I. Fluid circulation, mixing, and liquid velocity profiles. Biotechnol Bioeng 51:511–519

Wang SJ, Zhong JJ (1996b) A novel centrifugal impeller bioreactor II. Oxygen transfer and power consumption. Biotechnol Bioeng 51:511–519

Weathers PJ, Zobel RD (1992) Aeroponics for the culture of organisms, tissues and cells. Biotechnol Adv 10:93–115

Weathers PJ, Wyslouzil BE, Whipple M (1997) Laboratory-scale studies of nutrient mist reactors for culturing hairy roots. In: Doran PM (ed) Hairy Roots: Culture and Applications. Harwood Academic, Amsterdam, pp 191–199

Weathers PJ, Wyslouzil BE, Wobbe KK, Kim YJ, Yigit E (1999) The biological response of hair roots to O_2 levels in bioreactors. In Vitro Cell Dev Biol Plant 35:286–289

Whitney P (1992) Novel bioreactors for the growth of roots transformed by *Agrobacterium rhizogenes*. Enzyme Microb Technol 14:13–17

Widholm J (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol 47:189–194

Wilhelmson A, Häkkinen ST, Kallio PT, Oksman-Caldentey KM, Nuutila AM (2006) Heterologous expression of *Vitreoscilla* haemoglobin (VHb) and cultivation conditions affect the alkaloid profile of *Hyoscyamus muticus* hairy roots. Biotech Prog 22:350–358

Williams GRC, Doran PM (1999) Investigations of liquid–solid hydrodynamic boundary layers and oxygen requirements of hairy root cultures. Biotechnol Bioeng 64:729–740

Wilson PDG (1997) The pilot-scale cultivation of transformed roots. In: Doran PM (ed) Hairy Roots: Culture and Applications. Harwood Academic, Amsterdam, pp 179–190
Wink M, Alfermann AW, Franke R, Wetterauer B, Distl M, Windhoevel J, Krohn O, Fuss E, Garden H, Mohagheghzadeh A, Wildi E, Ripplinger P (2005) Sustainable production of phytochemicals by plant in vitro cultures: anticancer agents. Plant Gene Res 3:90–100
Withers LA (1991) Maintenance of plant tissue cultures. In: Kirsop BE, Doyle A (eds) Maintenance of Microorganisms, 2nd ed. Academic Press, London, pp 243–267
Wu J, Ge X (2004) Oxidative burst, jasmonic acid biosynthesis, and taxol production induced by low-energy ultrasound in *Taxus chinensis* cell suspension cultures. Biotechnol Bioeng 85:714–721
Wurm F (2005) Manufacture of recombinant biopharmaceutical proteins by cultivated mammalian cells in bioreactors. In: Knäblein J (ed) Modern Biopharmaceuticals, vol 3. Wiley VCH, Weinheim, pp 723–759
Xing HH, Ono A, Miyanaga K, Tanji Y, Unno H (2001) A kinetic model for growth of callus derived from *Eucommia ulmoides* aiming at mass production of a factor enhancing collagen synthesis of animal cells. Math Comput Simulat 56:463–474
Yanpaisan W, King N, Doran P (1998) Analysis of cell cycle activity and population dynamics in heterogeneous plant cell suspensions using flow cytometry. Biotechnol Bioeng 58:515–528.
Yanpaisan W, King N, Doran P (1999) Flow cytometry of plant cells with applications in large-scale bioprocessing. Biotechnol Adv 17:3–27
Yazaki K (2004) Natural products and metabolites. In: Christou P, Klee H (eds) Handbook of Plant Biotechnology, vol 2. Wiley, Chichester, pp 811–857
Ye H, Huang LL, Chen SD, Zhong JJ (2004) Pulsed electric field stimulates plant secondary metabolism in suspension cultures of *Taxus chinensis*. Biotechnol Bioeng 88:788–795
Yeoman MM, Yeoman CL (1996) Manipulating secondary metabolism in cultured plant cells. New Phytol 134:553–569
Yoshikawa T (1997) Production of ginsenosides in ginseng hairy root cultures. In: Doran PM (ed) Hairy Roots: Culture and Applications. Harwood Academic, Amsterdam, pp 73–79
Yoshimatsu K, Yamaguchi H, Shimomura K (1996) Traits of *Panax ginseng* hairy roots after cold storage and cryopreservation. Plant Cell Rep 15:555–560
Yu S, Mahagamasekera MGP, Willimas GRC, Kanokwaree K, Doran PM (1997) Oxygen effects in hairy root culture. In: Doran PM (ed) Hairy Roots: Culture and Applications. Harwood Academic, Amsterdam, pp 139–150
Zenk MH, El-Shagi H, Schulte U (1975) Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. Planta Med Suppl 79–101
Zenk MH, El-Shagi H, Arenes H, Stockgit J, Weiler EW, Dues D (1977) Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In: Barz W, Reinhard E, Zenk MH (eds) Plant Tissue Culture and its Biological Application. Springer, Berlin Heidelberg New York, pp 27–44
Zhang W, Curtin C, Kikuchi M, Franco C (2002) Integration of jasmonic acid and light irradiation for enhancement of anthocyanin biosynthesis in *Vitis vinifera* suspension cultures. Plant Sci 162:459–468
Zhong JJ (2001) Biochemical engineering of the production of plant-specific secondary metabolites. In: Tscheper T (ed) Advances in Biochemical Engineering: Plant Cells, vol 72. Springer, Berlin Heidelberg New York, pp 1–26
Zhong JJ, Yoshida M, Fujiyama K, Seki T, Yoshida T (1993) Enhancement of anthocyanin production by *Perilla frutescens* cells in a stirred bioreactor with internal light irradiation. J Ferment Bioeng 75:299–303
Zhong JJ, Chen F, Hu WW (1999) High density cultivation of *Panax notoginseng* cells in stirred bioreactors for the production of ginseng biomass and ginseng saponin. Process Biochem 35:491–496
Ziv M (2000) Bioreactor technology for plant micropropagation. In: Janick J (ed) Horticultural Reviews, vol 24. Wiley, New York, pp 1–30

Complementary Reading

Christou P, Klee H (2004) Handbook of Plant Biotechnology, vol 1–2. Wiley, Chichester
Endress R (1994) Plant Cell Biotechnology. Springer, Berlin Heidelberg New York
Evans D, Coleman J, Kearns A (2003) Plant Cell Culture. BIOS Scientific, Abingdon
Doran PM (1997) Hairy Roots: Culture and Applications. Harwood, Amsterdam
Dutta Gupta S, Ibaraki Y (2006) Plant Tissue Culture Engineering, Series: Focus on Biotechnology, vol 6. Springer, Dordrecht
Fischer R, Schillberg S (2004) Molecular Farming: Plant-made Pharmaceuticals and Technical Proteins. Wiley, Weinheim
Hess D (1992) Biotechnologie der Pflanzen. Eugen Ulmer, Stuttgart
Hvoslef-Eide AK, Preil W (2005) Liquid Culture Systems for In Vitro Plant Propagation. Springer, New York
IAEA (2004) Low cost options for tissue culture technology in developing countries. Proceedings of a Technical Meeting Organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, 26–30 August 2002
Oksman-Caldentey KM, Barz WH (2002) Plant Biotechnology and Transgenic Plants. Marcel Dekker, New York