World Journal of Microbiology & Biotechnology 11, 461–467

Recent advances in plant cell cultures in bioreactors

J.-J. Zhong,* J.-T. Yu and T. Yoshida

Two key issues in the application of plant-cell-culture technology to the production of valuable secondary metabolites are reviewed: the selection of cell lines with suitable genetic, biochemical and physiological characteristics; and the optimization of bioreactor environments. Although great progress has been made in recent years in the design, selection and optimization of bioreactor hardware, optimization of environmental factors such as medium components, light irradiation and O_2 supply needs detailed investigations for each case. With a better understanding of plant cell metabolism and physiology, further developments in cultivation processes, such as process integration and on-line monitoring and control, can be expected in the near future.

Key words: Bioprocess development, bioreactor optimization, environmental factors, modelling, monitoring and control, plant cell culture, secondary-metabolite production.

Although the history of plant cell culture, here taken as the culture of plant organs, tissue, cells, protoplast, embryos and plantlets, dates back to the beginning of this century, a great deal of progress has been achieved since the 1930s. The associated technology now has three main applications: the production of secondary metabolites; micropropagation; and the study of plant cell genetics, physiology, biochemistry and pathology. In this article, we review recent advances in the application of plant-cell-culture technology to metabolite production, new research on the optimization of bioreactor configurations and environmental factors and developments in plant cell bioprocesses.

Applications of Plant Cell Cultures to Production of Secondary Metabolites

Plant cell culture has several advantages as a method of producing useful metabolites. Plants produce more than 20,000 compounds, including pharmaceuticals, pigments and other fine chemicals, and this four times more than can be obtained from microbes. Some of these chemicals are difficult to synthesize chemically and it may also be difficult to produce them at all, or in significant amounts, using genetically engineered microorganisms. Cultures of plant cells are not limited by extrinsic environmental, ecological or climatic conditions and can therefore proliferate at higher growth rates than whole plants in cultivation. As shown in Table 1, some metabolites also accumulate at higher concentrations in cultured cells than in the parent plants. However, as the productivity of the cell cultures is low, their use is only economically viable if the metabolites are of high value, such as the anti-neoplastic drug, taxol, and related taxanes produced by *Taxus cuspidata* and *Tax. canadensis* callus and suspension cultures (Fett-Neto *et al.* 1992), and the new anti-malarial drug, artemisinin, produced in shoot cultures of *Artemisia annua* L. (Woerdenbag *et al.* 1993).

Generally, one main problem in the application of plant-cell-culture technology to secondary-metabolite production is a lack of basic knowledge about the biosynthetic routes and mechanisms regulating metabolite accumulation. However, there has been some recent progress in this field, in studies on elicitation, hairy-root culture, cell line modification through traditional and genetic engineering approaches, as well as the biochemistry.

Elicitation can effectively enhance metabolite synthesis in some cases, such as in thiophene production by hairy roots of *Tagetes patula* (Buitelaar *et al.* 1991) and tropane alkaloid production by suspension cultures of *Datura stramonium* cells (Ballica *et al.* 1993). Dunlop & Curtis (1991) also demonstrated that addition of fungal elicitors to hairy-root cultures of *Hyoscyamus muticus* enhanced the specific produc-

J.-J. Zhong and J.-T. Yu are with the Research Institute of Biochemical Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China; fax: 86 21 4776164. T. Yoshida is with the International Center of Cooperative Research in Biotechnology (ICBiotech), Faculty of Engineering, Osaka University, Suita, Osaka 565, Japan. *Corresponding author.

Table 1. Product yields from plant cell cultures compared with those of the parent plants.*

Product	Plant	Yield (% dry wt)		Yield ratio
		Culture	Plant	(culture/plant)
Anthocyanin	*Vitis* sp.	16	10	1.6
	Euphorbia milli	4	0.3	13.3
	Perilla frutescens	24	1.5	16.0
Anthraquinone	*Morinda citrifolia*	18	2.2	8.2
Berberine	*Coptis japonica*	13	4	3.3
	Thalictrum minor	10	0.01	1000
Rosmarinic acid	*Coleus blumei*	27	3	9.0
Shikonin	*Lithospermum erythrorhizon*	14	1.5	3.3

* From Zhong (1992).

tivity of solavetivone about 200-fold compared with that of non-elicited cultures. Increasing the activity of metabolic pathways by elicitation, in conjunction with end-product removal and accumulation in an extractive phase, has proven to be the most successful way of increasing metabolite productivity in general (Brodelius & Pedersen 1993).

The use of transformed roots is rapidly emerging as a viable alternative to the cultivation of whole plants for the production of plant secondary metabolites (Signs & Flores 1990; Toivonen *et al.* 1991; Christen *et al.* 1992). Transformed roots have inherent advantages over suspended-cell cultures and whole plant cultivation, including greater biochemical and genetic stability, faster growth rates than in whole plant cultivation, an alkaloid productivity reflecting that of the parent plant, and greater amenability to genetic manipulation. As the roots release a proportion of their intracellular products into the surrounding medium, continuous extraction is also possible. Hu & Alfermann (1993) used hairy-root cultures of *Salvia miltiorrhiza* for diterpenoid production and Toivonen *et al.* (1991) and Bhadra *et al.* (1993) successfully produced indole alkaloids, for possible vinblastine production, in hairy-root cultures of *Catharanthus roseus*.

Cell line selection is one of the traditional and effective approaches to enhancing metabolite accumulation. Wickremesinke & Arteca (1993), for example, established fast-growing callus and root cultures for potential production of harringtonine and related alkaloids. Genetic engineering is another promising tool for increasing the biosynthetic ability of plant cells. Hashimoto *et al.* (1993) increased scopolamine production in an *Atropa belladonna* hairy-root culture by expressing *Hyoscyamus niger* recombinant hyoscyamine-6-β-hydroxylase. In a model system, Gao & Lee (1992) demonstrated the efficient production of foreign protein (β-glucuronidase) by genetically modified tobacco cells.

Biochemical studies provide the fundamental information for the intentional regulation of secondary metabolism in plant cells. In a carrot suspension culture regulated by 2,4-dichlorophenoxyacetic acid, Ozeki *et al.* (1990) found that there was a correlation between anthocyanin synthesis and morphological differentiation for somatic embryogenesis. They also demonstrated that the induction and repression of phenylalanine ammonia lyase (PAL) and chalcone synthase correlated with formation of the respective mRNA. Two biosynthetic enzymes, PAL and 3-hydroxymethylglutaryl-CoA reductase, were also related to shikonin formation in *Lithospermum erythrorhizon* cultures (Srinivasan & Ryu 1992).

Although plant cell culture appears to be a useful method for the production of valuable secondary metabolites in the laboratory, many problems arise during bioprocess scale-up (Table 2). Thus there are only a few industrial-scale processes in operation, producing shikonin, phosphodiesterase, rosmarinic acid and ginseng. Whether or not more products produced in this way will reach the market largely depends on the economics of the process involved. This, in turn, is heavily dependent on the productivity of the culture. Selection of cell lines with suitable genetic, biochemical and physiological characteristics, is important. Optimization of bioreactor configurations and environmental conditions, which will be discussed later, is also definitely necessary to realize the commercial production of more useful metabolites by plant cells.

Optimization of Bioreactor Configurations and Culture Conditions

Design, Selection and Optimization of Bioreactor Hardware

Most of the bioreactors used to grow plant cells are directly derived from microbial fermenters. The choice and design of the most suitable reactor is determined by many factors, including shear environment, O_2-transfer capacity, mixing mechanism, the problem of foaming (Zhong *et al.* 1992b) and the need for aseptic conditions, all of which have to be tailored to the type of plant cells used and the purpose of the experiment. Understanding how to promote better cell culture through reactor modification, such as the use of impeller designs that produce reduced shear and the efficient use of light, is a major challenge (Treat *et al.* 1989).

Bioreactors of various types have been developed, including spin filter, continuously stirred turbine, hollow fibre,

Table 2. Problems in plant cell culture.

Biological	Operational
Slow growth rate	Wall adhesion
Physiological heterogeneity	Light requirement
Genetic instability	Viscosity
Low metabolite content	Shear sensitivity
Product secretion	Asepsis

two-step immobilization, stirred tank, air lift, rotating drum, and photo. Bioreactor modifications include replacing a flat-bladed turbine with a marine impeller or a single, large, flat paddle or blade to permit higher cell-growth rates (Treat *et al.* 1989; Hooker *et al.* 1990). Kim *et al.* (1991a), after developing a hybrid reactor with a cell-lift impeller and a sintered stainless-steel sparger for *Thalictrum rugosum* cell cultures, obtained cell densities of ≤ 31 g/l by perfusion, without any mixing problems or loss of cell viability; the specific berberine productivity was comparable with that in shake flasks. Su & Humphrey (1991) conducted a perfusion cultivation in a stirred-tank bioreactor fitted with an internal cross-flow filter which provided O_2 without bubble; a cell density of 26 g dry wt/l and a rosmarinic acid productivity of 94 mg/l/day were achieved. A double helical-ribbon impeller reactor with a working volume of 11 l was successfully developed for high-density cultivation of *Cat. roseus* cells (Jolicoeur *et al.* 1992). Yokoi *et al.* (1993) also developed a new type of stirred reactor, called a Maxblend fermenter, for high-density cultivation of plant cells, and they demonstrated its usefulness in cultivations of rice and shear-sensitive *Cat. roseus* cells.

Trickling film and 'mist' reactors, in which the roots are in contact with air most of the time and the medium is sprayed over the roots, have been used for root cultures (Whitney 1992). Hairy-root cultures of *Trigonella foenum-graceum* have been grown in modified 9-l airlift and 9-l column-mesh bioreactors (Rodriguez-Mendiola *et al.* 1991). Hairy-root cultures of *Datura stramonium* were grown in a stainless-steel cage inside a stirred-tank reactor for hyoscyamine production; the cage prevented direct contact between the roots and the stirrer and also provided a good support matrix, allowing a more even distribution of the roots in the reactor (Hilton & Rhodes 1990). There have also been reports on the use of bioreactors for immobilized plant-cell cultures (Archambault *et al.* 1990; Facchini & DiCosmo 1991). Kim & Chang (1990), for example, successfully used a dual hollow-fibre bioreactor to maintain high densities of immobilized *L. erythrorhizon* cells and continuous operation.

Optimization of Culture Environments

Medium Components. The effects of the medium components, both inorganic and organic, including hormones, employed in various plant cell cultures, such as the cultivation of the hairy roots of *Cat. roseus* (Bhadra *et al.* 1993) and suspended cells of *Coffea arabica* (Bramble *et al.* 1991), have been reported. A relatively high concentration of sucrose was reported to be favourable for rosmarinic acid production (Su & Humphrey 1990; Martinez & Park 1993). Carbon and nitrogen sources are often significant factors, affecting the accumulation of alkaloids by suspension cultures of *Holarrhena antidysenterica* (Panda *et al.* 1992), of anthocyanins by *Vitis vinifera* cell suspensions (Do & Cormier 1991), and of shikonin by *L. erythrorhizon* cell cultures (Srinivasan & Ryu 1993).

Light Irradiation. The spectral quality, intensity and period of light irradiation may all affect plant cell cultures in one way or another (Zhong *et al.* 1991). The stimulatory effect of light irradiation on the formation of several compounds, including anthocyanins, vindoline, catharanthine and thiophene, has been demonstrated (Kurata *et al.* 1991; Mukandan & Hjortso 1991; Zhong *et al.* 1991; Hirata *et al.* 1992). Zhong *et al.* (1991), who investigated the quantitative effect of light intensity on anthocyanin formation by *Perilla frutescens* cell cultures, found that 27.2 W/cm^2 favoured pigment production in a bioreactor.

Shear Stress. The effect of shear on biological cells has been investigated in various studies. Plant cells are usually sensitive to hydrodynamic stress as each usually has a large volume and a rigid, inflexible cell wall. Shear stress above a certain level reduces culture viability, cell mass and secondary-metabolite productivity, as demonstrated in cell-cultures of tobacco, *Cat. roseus* and *P. frutescens* (Scragg *et al.* 1988; Hooker *et al.* 1989; Leckie *et al.* 1991; Zhong *et al.* 1994a). However, different cell suspensions show different degrees of sensitivity to shear stress.

Oxygen Supply. O_2 supply affects both growth and metabolite production in a number of plant cell cultures, including those of *P. frutescens* (Zhong *et al.* 1993b) and *Cat. roseus* (Leckie *et al.* 1991). In flask cultures of *T. minus* cells, berberine-producing cells were observed to take up twice as much O_2 as non-producing cells (Kobayashi *et al.* 1991). Gao & Lee (1992) also demonstrated that an increase in the O_2 supply improved the specific O_2 uptake rate and the formation of a foreign protein (β-glucuronidase) and secondary metabolites (phenolics) in flask and bioreactor cultivations of tobacco cells. In contrast, O_2 starvation was claimed to stimulate pigment release in hairy-root cultures of red beet (Kino-oka *et al.* 1992).

Gas Composition. CO_2 and ethylene affect plant cell growth and metabolism in some cases. Both gases, for example, affect berberine formation in *T. minus* cell cultures; the specific berberine content was increased 2-fold when a

mixture of CO_2 and ethylene was added to an airlift system (Kim *et al.* 1991b). Gas composition was also found to be important in scale-up of the ajimalicine production process using *Cat. roseus* cultures (Schlatmann *et al.* 1993). Ethylene also affects root and shoot propagation and leaf-explant cultures of petunia (Dimasi-Therion *et al.* 1993).

Rheology. Knowledge of the rheology of plant cell cultures may help resolve various problems because culture viscosity, mixing, mass transfer, shear stress and cell growth, as well as metabolite production, all interact in a bioreactor cultivation (Zhong *et al.* 1992a). Cell cultures of *P. frutescens* were found to exhibit Bingham-plastic fluid characteristics, and the size of the individual cells, not the cell aggregates, affected the cultures' rheological characteristics (Zhong *et al.* 1992a). Ballica *et al.* (1992) studied the rheological properties and determined the yield stress value of *Dat. stramonium* cell suspensions, factors considered to be helpful in the bioprocess engineering of plant cells for high density, particularly in determining reactor operating strategies. Curtis & Emery (1993), investigating the rheological characteristics of 10 different plant-cell suspension cultures, claimed that most plant cell suspensions displayed Newtonian behaviour at moderate cell densities and that the relatively rare non-Newtonian behaviour was a result of cellular elongation.

Advances in Bioreactor Cultivation Processes

Continuous Culture

Van Gulik *et al.* (1992) investigated the use of a chemostat culture technique to obtain reliable data on the stoichiometry of the growth of plant cells in a stirred tank reactor. Several other groups have also studied the growth kinetics and stoichiometry and modelled the growth of suspension-cultured plant cells, using semi-continuous or fed-batch cultures to achieve steady-state growth. Westgate *et al.* (1991), for example, presented fed-batch cultivation kinetics for continuous approximation in *Cephalotaxus harringtonia* cultures.

Two-stage Culture

The most well-known example of two-stage culture is that adopted by the Japanese Mitsui Petrochemical Company in the commercial production of shikonin. In a study of biotransformation by plant cells, Kreis & Reinhard (1990) developed a process in which *Digitalis lanata* cells were first propagated in a growth medium and then transferred to the appropriate production medium, where the cells converted digitoxin into 12-β-hydroxylated products. Jung *et al.* (1994) also utilized a two-stage culture process, for hairy-root cultures of *Cat. roseus*, optimizing the inorganic salts and enhancing catharanthine productivity up to 5.4-fold compared with that in a one-stage culture.

Cell Immobilization

There have been many publications on the immobilization of plant cells since the first report in 1979 (Brodelius *et al.* 1979) and the methods now available include gel entrapment, adsorption, and foam (e.g. polyurethane) immobilization. The possible advantages of immobilization include the ability to use continuous-flow processes, the easy separation of biocatalysts from the reaction medium, the cell-to-cell contact, which may be beneficial to secondary metabolite synthesis, and the protection of sensitive plant cells against shear stress. Some potential problems are the introduction of gradients in the gel beads which are often used, the necessity for product excretion, and loss of cell viability in many cases.

Secretion of secondary metabolites is a pre-requisite for cell immobilization. Several methods, such as temperature adjustment, electrical permeabilization, altering medium composition, and permeabilization with chemicals such as dimethylsulphoxide (DMSO), can be used to improve product recovery (Buitelaar & Tramper 1992). Park & Martinez (1992) reported a new approach to plant-cell permeabilization in which DMSO treatment was coupled with preconditioning; this resulted in substantial rosmarinic acid secretion by and a high viability of permeabilized *Coleus blumei* cells.

Process Integration

Two-phase culture is used to selectively remove the desired product from a reactor. One phase is the aqueous medium and the second either a water-immiscible organic solvent or a solid compound. Kim & Chang (1990) reported that *in situ* extraction and immobilization greatly increased cellular and volumetric shikonin productivities. The isolation of shikonin by *in situ* extraction, with *n*-hexadecane, was also studied in hairy-root cultures in shake-flask cultures and a bubble column (Sim & Chang 1993). Buitelaar *et al.* (1991) observed good growth and thiophene production in hairy-root cultures of *Tagetes patula* in various two-liquid-phase bioreactors. Similarly, Byun *et al.* (1992) used a compounded silicone-fluid two-phase culture system to enhance production of sanguinarine by *Eschscholtzia california.*

Process Monitoring, Modelling and Control

In spite of a great need for better monitoring and control in the optimization of plant cell bioprocesses, few studies have been published in this area. The monitoring parameters most frequently reported in plant cell cultures are the concentrations of cells and NAD(P)H (Asali *et al.* 1992) in the reactor and of O_2/CO_2 in the inlet and outlet gases (Rho *et al.* 1990; Zhong *et al.* 1994b). Cell concentration is monitored as conductivity (Taya *et al.* 1989b; Ryu *et al.* 1990), osmotic pressure (Tanaka *et al.* 1993), dielectric (Markx *et al.* 1991) or turbidimetry (Tanaka *et al.* 1992; Zhong *et al.* 1993a). For example, Zhong *et al.* (1993a)

showed that the redness of the anthocyanin in *P. frutescens* cell cultures did not interfere with measurement of the turbidity at 780 nm, using a laser sensor, and succeeded in the real-time *in situ* monitoring of the cell mass in a stirred bioreactor. Furthermore, a computer-aided, on-line, real-time monitoring system for plant cell processes was established and applied to the cultivation of *P. frutescens* cells in the bioreactor (Zhong *et al.* 1994b). The system was found to be useful for the identification of the physiological states (such as the respiratory quotient and specific O_2 uptake rate) of the plant cells during cultivation. In studies of somatic embryogenesis, Cazzulino *et al.* (1990) classified carrot somatic embryos using an image analyzer, and Hamalainen *et al.* (1993) presented specific features suitable for the classification of birch somatic embryos and developed a classifier using these features for possible automatic processing.

Mathematical models of biological processes are often used for hypothesis testing and process optimization. Using physical interpretations of results to obtain greater insights into process behaviour is only possible when structured models, in which several parts of the system are considered separately, are employed. Several dynamic mathematical models of plant cell growth and metabolite production have been developed (Bailey & Nicholson 1989; Bramble *et al.* 1991; Curtis *et al.* 1991; Hooker & Lee 1992; Van Gulik *et al.* 1993). Hooker & Lee (1992) produced a basic structured kinetic model, applicable to batch suspension cultures of tobacco, in which the interactions between structural component production, secondary metabolite synthesis and cellular respiration are considered. In characterizing the hairy-root growth of carrot (*Daucus carota*), horseradish (*Armoracia lapathifolia*), senna (*Cassia torosa*) and pak-bung (*Ipomoea aquatica*), Taya *et al.* (1989a) proposed a kinetic model based on the linear extension and lateral branching of the growing point at the root tip. Cazzulino *et al.* (1990) proposed a segregated kinetic model to describe substrate utilization, culture growth, and embryo development, in an embryogenic culture of carrot, in a rigorous, quantitative manner.

Several interesting parameter-control models or systems have been reported in recent years: a five-state mathematical model for temperature control (Bailey & Nicholson 1990); a mathematical-model description of the phenomenon of light absorption by *Cof. arabica* suspension-cell cultures in a photo-culture vessel (Kurata & Furusaki 1993); and a bioreactor control system for the simultaneous control of the concentrations of dissolved O_2 and CO_2 (Smith *et al.* 1990). In addition, a physiological-state control approach, in which the current physiological state of a cell culture is monitored (Zhong *et al.* 1994b), may be a powerful method for the control of plant cell processes, because, being based on artificial intelligence methods, particularly fuzzy sets and pattern recognition theory, no conventional mathematical model is required for the synthesis of such a control system.

There is reason to believe that great advances in process control and the optimization of plant cell cultures will be achieved in the relatively near future. At present, there are two main obstacles in these research areas: the lack of an adequate on-line process monitoring system for plant cells; and the heterogeneity and instability of the cells. However, it has been demonstrated that the first problem can be solved and we expect that the recent developments in plant cell biology, particularly those in biochemistry and molecular biology, will soon help to resolve the second. Close co-operation between biologists and biochemical engineers is necessary and both groups must expand their fields of knowledge and research fields to create a common cutting-edge.

Acknowledgements

The authors' research described in the review was conducted at ICBiotech, Osaka University. Preparation of the manuscript was supported by a grant to JJZ from the Foundation of State Education Commission of China for Returned Scholars from Abroad.

References

Archambault, J., Volesky, B. & Kurz, W.G.W. 1990 Development of bioreactors for the culture of surface immobilized plant cells. *Biotechnology and Bioengineering* **35**, 702–711.

Asali, E.C., Mutharasan, R. & Humphrey, A.E. 1992 Use of NAD(P)H-fluorescence for monitoring the response of starved cells of *Catharanthus roseus* in suspension to metabolic perturbations. *Journal of Biotechnology* **23**, 83–94.

Bailey, C.M. & Nicholson, H. 1989 A new structured model for plant cell culture. *Biotechnology and Bioengineering* **34**, 1331–1336.

Bailey, C.M. & Nicholson, H. 1990 Optimal temperature control for a structured model of plant cell culture. *Biotechnology and Bioengineering* **35**, 252–259.

Ballica, R., Ryu, D.D.Y. & Kado, C.I. 1993 Tropane alkaloid production in *Datura stramonium* suspension cultures: elicitor and precursor effects. *Biotechnology and Bioengineering* **41**, 1075–1081.

Ballica, R., Ryu, D.D.Y., Powell, R.L. & Owen, D. 1992 Rheological properties of plant cell suspensions. *Biotechnology Progress* **8**, 413–420.

Bhadra, R., Vani, S. & Shanks, J.V. 1993 Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus*. *Biotechnology and Bioengineering* **41**, 581–592.

Bramble, J.L., Graves, D.J. & Brodelius, P. 1991 Calcium and phosphate effects on growth and alkaloid production in *Coffea arabica*: experimental results and mathematical model. *Biotechnology and Bioengineering* **37**, 859–868.

Brodelius, P., Deus, B., Mosbach, K. & Zenk, M.H. 1979 Immobilized plant cells for the production and transformation of natural products. *FEBS Letters* **103**, 93–97.

Brodelius, P. & Pedersen, H. 1993 Increasing secondary metabolite production in plant-cell culture by redirecting transport. *Trends in Biotechnology* **11**, 30–36.

Buitelaar, R.M., Langenhoff, A.A.M., Heidstra, R. & Tramper, J.

1991 Growth and thiophene production by hairy root cultures of *Tagetes patula* in various two-liquid-phase bioreactors. *Enzyme and Microbial Technology* **13**, 487–494.

Buitelaar, R.M. & Tramper, J. 1992 Strategies to improve the production of secondary metabolites with plant cell cultures: a literature review. *Journal of Biotechnology* **23**, 111–141.

Byun, S.Y., Ryu, Y.W., Kim, C. & Pedersen, H. 1992 Elicitation of sanguinarine production in two-phase cultures of *Eschscholtzia california*. *Journal of Fermentation and Bioengineering* **73**, 380–385.

Cazzulino, D.L., Pedersen, H., Chin, C.-K. & Styer, D. 1990 Kinetics of carrot somatic embryo development in suspension culture. *Biotechnology and Bioengineering* **35**, 781–786.

Christen, P., Aoki, T. & Shimomura, K. 1992 Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *A. rhizogenus* A4. *Plant Cell Reports* **11**, 597–600.

Curtis, W.R. & Emery, A.H. 1993 Plant cell suspension culture rheology. *Biotechnology and Bioengineering* **42**, 520–526.

Curtis, W.R., Hasegawa, P.M. & Emery, A.H. 1991 Modeling linear and variable growth in phosphate limited suspension cultures of opium poppy. *Biotechnology and Bioengineering* **38**, 371–379.

Dimasi-Therion, K., Economon, A.S. & Sfakiotakis, E.M. 1993 Promotion of petunia (*Petunia hybrida* L.) regeneration *in vitro* by ethylene. *Plant Cell, Tissue and Organ Culture* **32**, 219–255.

Do, C.B. & Cormier, F. 1991 Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (*Vitis vinifera* L.) cell suspension. *Plant Cell Reports* **9**, 500–504.

Dunlop, D.S. & Curtis, W.R. 1991 Synergistic response of plant hairy-root cultures to phosphate limitation and fungal elicitation. *Biotechnology Progress* **7**, 434–438.

Facchini, P.J. & DiCosmo, F. 1991 Plant cell bioreactor for the production of protoberberine alkaloids from immobilized *Thalictrum rugosum* cultures. *Biotechnology and Bioengineering* **37**, 397–403.

Fett-Neto, A.G., DiCosmo, F., Reynolds, W.F. & Sakata, K. 1992 Cell culture of *Taxus* as a source of the antineoplastic drug taxol and related taxanes. *Bio/Technology* **10**, 1572–1575.

Gao, J. & Lee, J.M. 1992 Effect of oxygen supply on the suspension culture of genetically modified tobacco cells. *Biotechnology Progress* **8**, 285–290.

Hamalainen, J.J., Kurten, U. & Kauppinen, V. 1993 Classification of plant somatic embryos by computer vision. *Biotechnology and Bioengineering* **41**, 35–42.

Hashimoto, T., Yun, D.J. & Yamada, Y. 1993 Production of tropane alkaloids in genetically engineered root cultures. *Phytochemistry* **32**, 713–718.

Hilton, M.G. & Rhodes, M.J.C. 1990 Growth and hyoscyamine production of hairy root cultures of *Datura stramonium* in a modified stirred tank reactor. *Applied Microbiology and Biotechnology* **33**, 132–138.

Hirata, K., Horiuchi, M., Asada, M., Ando, T., Miyamoto, K. & Miura, Y. 1992 Stimulation of dimeric alkaloid production by near-ultraviolet light in multiple shoot cultures of *Catharanthus roseus*. *Journal of Fermentation and Bioengineering* **74**, 222–225.

Hooker, B.S. & Lee, J.M. 1992 Application of a new structured model to tobacco cell cultures. *Biotechnology and Bioengineering* **39**, 765–774.

Hooker, B.S., Lee, J.M. & An, G. 1989 Response of plant tissue culture to a high shear environment. *Enzyme and Microbial Technology* **11**, 484–490.

Hooker, B.S., Lee, J.M. & An, G. 1990 Cultivation of plant cells in a stirred vessel: effect of impeller design. *Biotechnology and Bioengineering* **35**, 296–304.

Hu, Z.B. & Alfermann, A.W. 1993 Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. *Phytochemistry* **32**, 699–703.

Jolicoeur, M., Chavarie, C., Carreau, P.J. & Archambault, J. 1992 Development of a helical-ribbon impeller bioreactor for high-density plant cell suspension culture. *Biotechnology and Bioengineering* **39**, 511–521.

Jung, K.-H., Kwak, S.-S., Choi, C.-Y. & Liu, J.R. 1994 Development of two stage culture process by optimization of inorganic salts for improving catharanthine production in hairy root cultures of *Catharanthus roseus*. *Journal of Fermentation and Bioengineering* **77**, 57–61.

Kim, D.-I., Cho, G.H., Pedersen, H. & Chin, C.-K. 1991a A hybrid bioreactor for high density cultivation of plant cell suspensions. *Applied Microbiology and Biotechnology* **34**, 726–729.

Kim, D.-I., Pedersen, H. & Chin, C.-K. 1991b Cultivation of *Thalictrum rugosum* cell suspension in an improved airlift bioreactor: stimulatory effect of carbon dioxide and ethylene on alkaloid production. *Biotechnology and Bioengineering* **38**, 331–339.

Kim, D.J. & Chang, H.N. 1990 Enhanced shikonin production from *Lithospermum erythrorhizon* by *in situ* extraction and calcium alginate immobilization. *Biotechnology and Bioengineering* **36**, 460–466.

Kino-oka, M., Hongo, Y., Taya, M. & Tone, S. 1992 Culture of red beet hairy root in bioreactor and recovery of pigment released from the cells by repeated treatment of oxygen starvation. *Journal of Chemical Engineering of Japan* **25**, 490–495.

Kobayashi, Y., Fukui, H. & Tabata, M. 1991 Effect of carbon dioxide and ethylene on berberine production and cell browning in *Thalictrum minus* cell cultures. *Plant Cell Reports* **9**, 496–499.

Kreis, W. & Reinhard, E. 1990 Two-stage cultivation of *Digitalis lanata* cells: semicontinuous production of deacetyllanatoside C in 20-litre airlift bioreactors. *Journal of Biotechnology* **16**, 123–136.

Kurata, H. & Furusaki, S. 1993 Nonisotropic scattering model for estimation of light absorption rates in a suspension culture of *Coffea arabica* cells. *Biotechnology Progress* **9**, 86–92.

Kurata, H., Seki, M., Furusaki, S. & Furuya, T. 1991 Influence of light irradiation rates and irradiation modes on caffeine production and cell growth in suspension culture of *Coffea arabica* cells. *Journal of Chemical Engineering of Japan* **24**, 783–788.

Leckie, F., Scragg, A.H. & Cliffe, K.C. 1991 Effect of bioreactor design and agitator speed on the growth and alkaloid accumulation by cultures of *Catharanthus roseus*. *Enzyme and Microbial Technology* **13**, 296–305.

Markx, G.H., Davey, C.L., Kell, D.B. & Morris, P. 1991 The dielectric permittivity at radio frequencies and the Bruggeman probe: novel techniques for the on-line determination of biomass concentrations in plant cell cultures. *Journal of Biotechnology* **20**, 279–290.

Martinez, B.C. & Park, C.-H. 1993 Characteristics of batch suspension cultures of preconditioned *Coleus blumei* cells: sucrose effect. *Biotechnology Progress* **9**, 97–100.

Mukandan, U. & Hjortso, M.A. 1991 Effect of light on growth and thiophene accumulation in transformed roots of *Tagetes patula*. *Journal of Plant Physiology* **138**, 252–255.

Ozeki, Y., Komamine, A. & Tanaka, Y. 1990 Induction and repression of phenylalanine ammonia-lyase and chalcone synthase enzyme proteins and mRNAs in carrot cell suspension cultures regulated by 2,4-D. *Physiologia Plantarum* **78**, 400–408.

Panda, A.K., Mishra, S. & Bisaria, V.S. 1992 Alkaloid production by plant cell suspension cultures of *Holarrhena antidysenterica*: (I) effect of major nutrients. *Biotechnology and Bioengineering* **39**, 1043–1051.

Park, C.-H. & Martinez, B.C. 1992 Enhanced release of rosmarinic acid from *Coleus blumei* permeabilized by dimethyl sulfoxide (DMSO) while preserving cell viability and growth. *Biotechnology and Bioengineering* **40**, 459–464.

Rho, D., Bedard, C. & Archambault, J. 1990 Physiological aspects of surface-immobilized *Catharanthus roseus* cells. *Applied Microbiology and Biotechnology* **33**, 59–65.

Rodriguez-Mendiola, M.A., Stafford, A., Cresswell, R. & Arias-Castro, C. 1991 Bioreactors for growth of plant roots. *Enzyme and Microbial Technology* **13**, 697–702.

Ryu, D.D.Y., Lee, S.O. & Romani, R.J. 1990 Determination of growth rate for plant cell cultures: comparative studies. *Biotechnology and Bioengineering* **35**, 305–311.

Schlatmann, J.E., Nuutila, A.M., Van Gulik, W.M., Ten Hoopen, H.J.G., Verpoorte, R. & Heijnen, J.J. 1993 Scaleup of ajmalicine production by plant cell cultures of *Catharanthus roseus*. *Biotechnology and Bioengineering* **41**, 253–262.

Scragg, A.H., Allan, E.J. & Leckie, F. 1988 Effect of shear on the viability of plant cell suspensions. *Enzyme and Microbial Technology* **10**, 361–367.

Signs, M. & Flores, H. 1990 The biosynthetic potential of plant roots. *BioEssays* **12**, 7–13.

Sim, S.J. & Chang, H.N. 1993 Increased shikonin production by hairy roots of *Lithospermum erythrorhizon* in two-phase bubble column reactor. *Biotechnology Letters* **15**, 145–150.

Smith, J.M., Davison, S.W. & Payne, G.F. 1990 Development of a strategy to control the dissolved concentrations of oxygen and carbon dioxide at constant shear in a plant cell bioreactor. *Biotechnology and Bioengineering* **35**, 1088–1101.

Srinivasan, V. & Ryu, D.D.Y. 1992 Enzyme activity and shikonin production in *Lithospermum erythrorhizon* cell cultures. *Biotechnology and Bioengineering* **40**, 69–74.

Srinivasan, V. & Ryu, D.D.Y. 1993 Improvement of shikonin productivity in *Lithospermum erythrorhizon* cell culture by alternating carbon and nitrogen feeding strategy. *Biotechnology and Bioengineering* **42**, 793–799.

Su, W.W. & Humphrey, A.E. 1990 Production of rosmarinic acid in high density perfusion cultures of *Anchusa officinalis* using a high sugar medium. *Biotechnology Letters* **12**, 793–798.

Su, W.W. & Humphrey, A.E. 1991 Production of rosmarinic acid from perfusion culture of *Anchusa officinalis* in a membrane-aerated bioreactor. *Biotechnology Letters* **13**, 889–892.

Tanaka, H., Aoyagi, H. & Jitsufuchi, T. 1992 Turbidimetric measurement of cell biomass of plant cell suspensions. *Journal of Fermentation and Bioengineering* **73**, 130–134.

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. *Journal of Fermentation and Bioengineering* **76**, 501–504.

Taya, M., Kino-oka, M., Tone, S. & Kobayashi, T. 1989a A kinetic model of branching growth of plant hairy root. *Journal of Chemical Engineering of Japan* **22**, 698–700.

Taya, M., Tone, S. & Prenosil, J.E. 1989b Plant cell culture by medium circulating bioreactor and on-line estimation of cell mass. *Plant Tissue Culture Letters (Japan)* **6**, 179–181.

Toivonen, L., Ojala, M. & Kauppinen, V. 1991 Studies on the optimization of growth and indole alkaloid production by hairy root cultures of *Catharanthus roseus*. *Biotechnology and Bioengineering* **37**, 673–680.

Treat, W.J., Engler, C.R. & Soltes, E.J. 1989 Culture of photomixotrophic soybean and pine in a modified fermenter using a novel impeller. *Biotechnology and Bioengineering* **34**, 1191–1202.

Van Gulik, W.M., Ten Hoopen, H.J.G. & Heijnen, J.J. 1992 Kinetics and stoichiometry of growth of plant cell cultures of *Catharanthus roseus* and *Nicotiana tabacum* in batch and continuous fermenters. *Biotechnology and Bioengineering* **40**, 863–874.

Van Gulik, W.M., Ten Hoopen, H.J.G. & Heijnen, J.J. 1993 A structured model describing carbon and phosphate limited growth of *Catharanthus roseus* plant cell suspensions in batch and chemostat culture. *Biotechnology and Bioengineering* **41**, 771–780.

Westgate, P.J., Curtis, W.R., Emery, A.H., Hasegawa, P.M. & Heinstein, P.F. 1991 Approximation of continuous growth of *Cephalotaxus harringtonia* plant cell cultures using fed-batch operation. *Biotechnology and Bioengineering* **38**, 241–246.

Whitney, P.J. 1992. Novel bioreactors for the growth of roots transformed by *Agrobacterium rhizogenes*. *Enzyme and Microbial Technology* **14**, 13–17.

Wickremesinke, E.R. & Arteca, R.N. 1993 Establishment of fast-growing callus and root cultures of *Cephalotaxus harringtonia*. *Plant Cell Reports* **12**, 80–83.

Woerdenbag, H.J., Luers, J.F.J., Van Uden, W., Pras, N., Malingre, T.M. & Alfermann, A.W. 1993 Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. *Plant Cell, Tissue and Organ Culture* **32**, 247–257.

Yokoi, H., Koga, J., Yamamoto, K., Seike, Y., Tanaka, H. 1993 High density cultivation of plant cells in a new aeration-agitation type fermenter, Maxblend Fermenter. *Journal of Fermentation and Bioengineering* **75**, 48–52.

Zhong, J.-J. 1992 Bioprocess engineering studies on suspended cultures of *Perilla frutescens* in bioreactors for anthocyanin production. PhD Thesis. Osaka University, Japan.

Zhong, J.-J., Fujiyama, K., Seki, T. & Yoshida, T. 1993a On-line monitoring of cell concentration of *Perilla frutescens* in a bioreactor. *Biotechnology and Bioengineering* **42**, 542–546.

Zhong, J.-J., Fujiyama, K., Seki, T. & Yoshida, T. 1994a A quantitative analysis of shear effects on cell suspension and cell culture of *Perilla frutescens* in bioreactors. *Biotechnology and Bioengineering* **44**, 649–654.

Zhong, J.-J., Konstantinov, K.B. & Yoshida, T. 1994b Computer-aided on-line monitoring of physiological variables in suspended cell cultures of *Perilla frutescens* in a bioreactor. *Journal of Fermentation and Bioengineering* **77**, 445–447.

Zhong, J.-J., Seki, T., Kinoshita, S. & Yoshida, T. 1991 Effect of light irradiation on anthocyanin production by suspended culture of *Perilla frutescens*. *Biotechnology and Bioengineering* **38**, 653–658.

Zhong, J.-J., Seki, T., Kinoshita, S. & Yoshida, T. 1992a Rheological characteristics of cell suspension and cell culture of *Perilla frutescens*. *Biotechnology and Bioengineering* **40**, 1256–1262.

Zhong, J.-J., Seki, T., Kinoshita, S. & Yoshida, T. 1992b Effects of surfactants on cell growth and pigment production in suspension cultures of *Perilla frutescens*. *World Journal of Microbiology and Biotechnology* **8**, 106–109.

Zhong, J.-J., Yoshida, M., Fujiyama, K., Seki, T. & Yoshida, T. 1993b Enhancement of anthocyanin production by *Perilla frutescens* cells in s stirred bioreactor with internal light irradiation. *Journal of Fermentation and Bioengineering* **75**, 299–303.