Appl Microbiol Biotechnol (2007) 74:1175–1185
DOI 10.1007/s00253-007-0856-5

MINI-REVIEW

Hairy root type plant in vitro systems as sources of bioactive substances

Milen I. Georgiev · Atanas I. Pavlov · Thomas Bley

Received: 30 October 2006 / Revised: 15 January 2007 / Accepted: 21 January 2007 / Published online: 10 February 2007

Abstract "Hairy root" systems, obtained by transforming plant tissues with the "natural genetic engineer" *Agrobacterium rhizogenes*, have been known for more than three decades. To date, hairy root cultures have been obtained from more than 100 plant species, including several endangered medicinal plants, affording opportunities to produce important phytochemicals and proteins in eco-friendly conditions. Diverse strategies can be applied to improve the yields of desired metabolites and to produce recombinant proteins. Furthermore, recent advances in bioreactor design and construction allow hairy root-based technologies to be scaled up while maintaining their biosynthetic potential. This review highlights recent progress in the field and outlines future prospects for exploiting the potential utility of hairy root cultures as "chemical factories" for producing bioactive substances.

Keywords *Agrobacterium rhizogenes* · Hairy roots · Secondary metabolites · Bioreactors

M. I. Georgiev (✉) · T. Bley
Institute of Food Technology and Bioprocess Engineering,
Dresden University of Technology,
01069 Dresden, Germany
e-mail: Milen.Georgiev@mailbox.tu-dresden.de

A. I. Pavlov
Department of Microbial Biosynthesis and Biotechnologies—
Laboratory in Plovdiv,
Institute of Microbiology,
Bulgarian Academy of Sciences,
26 Maritza Blvd.,
4002 Plovdiv, Bulgaria

Introduction

From time immemorial, mankind has been highly dependent on plants as sources of proteins, carbohydrates and fats (Rao and Ravishankar 2002). Furthermore, the volume and range of phytochemicals used by modern society (inter alia as drugs, nutrients, cosmetic additives and biopesticides) are continuously expanding. Currently, more than 25% of all prescribed medicines used in industrialized countries are derived either directly or indirectly from plants, and annual sales of these products in the USA alone exceeded 30 billion dollars in 2002 (Raskin et al. 2002; Fowler 2006). These high demands are driving efforts to develop new ways to produce plant-derived metabolites.

Plant in vitro techniques, in which plant cells, tissue and organs are cultivated in sterile conditions totally independently of geographical and climatic factors, offer such alternatives for producing important metabolites, and several technologies based on them have been developed (Kieran 2001). The exploitation of transformed root cultures (also called "hairy roots," by analogy with the natural plant disease of the same name, which causes massive production of adventitious roots with numerous root hairs) presents a relatively novel approach to in vitro plant biotechnology that has received increasing attention in recent years. Although the mechanism of *Agrobacterium*-mediated genetic transformation has been known for more than 30 years, the enormous capacity of transformed root cultures has been largely neglected for most of that time. However, in the mid-1980s, hints of the biosynthetic potential of hairy roots were obtained through a series of investigations, focusing mainly on their alkaloids production (Mano et al. 1986; Payne et al. 1987). During the 1990s, research in this field entered an "exponential phase," and more than 400 papers on hairy roots were published

during this decade (source: SCOPUS database). The published studies highlighted several advantages of transformed root cultures, including their relatively fast growth rates (in hormone-free media), genetic and biochemical stability and capacity for organogenesis-associated synthesis of metabolites (Shanks and Morgan 1999; Sevon and Oksman-Caldentey 2002). In addition, recent developments in bioreactor systems provide ways in which it may be possible to scale up hairy root cultivation from small-scale systems to large-scale industrial processes (Guillon et al. 2006a). Furthermore, growing interest in hairy root systems from private companies (e.g. ROOTec bioactives GmbH, Basel, Switzerland), should facilitate the transfer of knowledge from academic laboratories to the pharmaceutical, food and cosmetic industries.

The present review summarizes recent developments in hairy roots research, including methods for mother plant transformations, the production of valuable biological active substances and strategies for enhancing the yields of desired metabolites. It also considers recent advances in bioreactor designs for hairy root cultivation and scaling-up processes.

Historical background and establishment

At the beginning of the last century, two plant diseases, crown-gall and hairy root, caused significant losses in vineyards, plum, apple and peach nurseries, and several investigations were initiated to elucidate their nature and the infection mechanisms involved. Several years later, the molecular mechanisms underlying these diseases were elucidated and shown to be based on the natural genetic engineering abilities of phytopathogenic soil bacteria of the genus *Agrobacterium* (*Rhizobiaceae* family; Chilton et al. 1977, 1980). When various susceptible plants (mainly gymnosperms and dicotyledon species) are wounded in natural ecosystems or fields, they produce simple phenolic substances, such as acetosyringone (Fig. 1), which induces the plasmid-localized *vir*(virulence) genes of *Agrobacterium* that are responsible for transferring the T-DNA fragments of the Ti-(tumor inducing) plasmid or Ri-(root inducing) plasmid (in the cases of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively) to the plant cells (Nilsson and Olsson 1997). Genes of the transferred DNA fragment mediate the formation of neoplastic crown gall tumors and hairy root tissues, which produce (and secrete into the soil) substances called opines (unusual amino acids; Fig. 1), further used by *Agrobacterium* as nutrients (Weising and Kahl 1996). Most *Agrobacterium* strains contain only one T-DNA, but some (like those carrying agropine type Ri-plasmids) transfer two independent T-DNAs, denoted T_L-DNA and T_R-DNA. T_R-DNA has high homology to the T-DNA of the Ti-plasmid

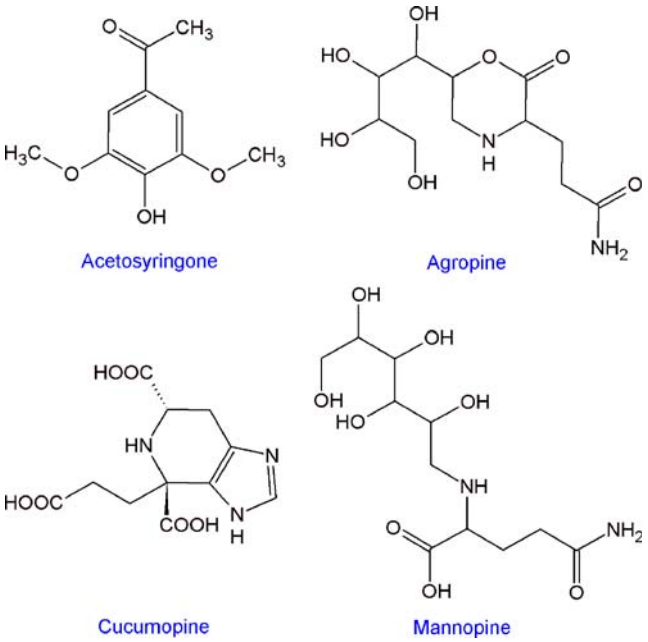

Fig. 1 Chemical structures of acetosyringone and some opines

of *A. tumefaciens*, while T_L-DNA is strikingly different and has homology to the T-DNA carried by the Ri-plasmid of mannopine *A. rhizogenes* strains (Nilsson and Olsson 1997). Both T_L-DNA and T_R-DNA are transferred and integrated independently into the host plant genome, but the transfer of T_L-DNA is essential for induction of the hairy root syndrome, and transfer of T_R-DNA does not provoke formation of roots from transformed cultures (Nilsson and Olsson 1997; Sevon and Oksman-Caldentey 2002). T_R-DNA contains two genes, *iaaM* and *iaaH*, responsible for the biosynthesis of auxins and the genes responsible for the synthesis of the opines mannopine (*mas1′* and *mas2′*) and agropine (*ags*). T_L-DNA carries 18 open reading frames (ORF), four of which are essential for hairy root formation; ORF10, ORF11, ORF12 and ORF15 corresponding to the genes *rol*A, *rol*B, *rol*C and *rol*D, respectively. The *rol*B gene is absolutely essential for the induction of hairy roots. Even when expressed alone, the *rol*B gene can induce significant hairy root production (Nilsson and Olsson 1997). Detailed information regarding the mechanism involved in the genetic transfer can be found in Gelvin (2000) and Tzfira et al. (2004).

This natural phenomenon is exploited in biotechnology to generate transformed root cultures. The procedure used to induce hairy roots (Fig. 2) includes cultivation of wounded plant parts (called explants) with suspensions of *A. rhizogenes* in aseptic conditions (Sevon and Oksman-Caldentey 2002). Wounded plant explants can be infected with *Agrobacterium* strains either by direct inoculation with bacterial suspensions and incubation on a solid medium or by co-cultivation in liquid media. In either case, the infected explants have to be subsequently transferred to a

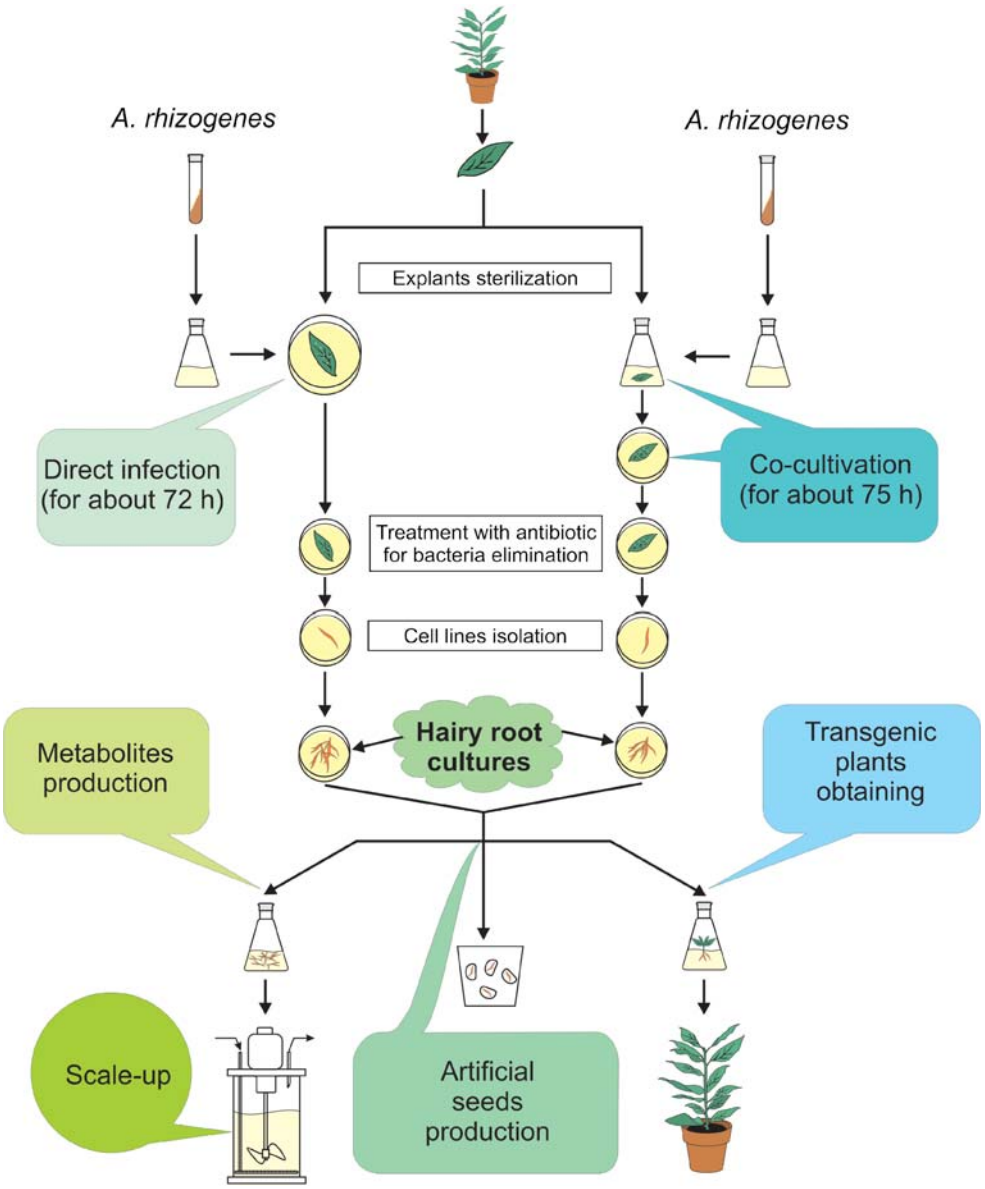

Fig. 2 Flow chart for the induction of hairy root cultures and some of their applications

solid medium with antibiotics (claforan or penicillin derivates), usually about 72 h later, to eliminate the bacteria (Pavlov et al. 2002; Rahman et al. 2004). The neoplastic, transformed roots (which usually appear after 1–4 weeks) grow in a profusely branched manner, with abundant lateral branches, on Murashige and Skoog or Gamborg's B5 hormone-free media (Pavlov et al. 2002; Sevon and Oksman-Caldentey 2002). Successful genetic transformation can be demonstrated in either of two ways: directly or indirectly, by detecting T-DNA or opines, respectively. The direct way is preferred, as in some cases, opine production is not stable and may even cease (Sevon and Oksman-Caldentey 2002). To detect T-DNA, either polymerase chain reactions (Palazon et al. 2003a; Le Flem-Bonhomme et al. 2004) or Southern blot hybridizations (Nin et al. 1997; Xie et al. 2001) can be used. After a short period of adaptation, the hairy root cultures can then be used to produce metabolites (Wysokinska and Chmiel 1997; Shanks and Morgan 1999; Sevon and Oksman-Caldentey 2002), artificial seeds (Uozumi 2004) or transgenic plants (Choi et al. 2004; Crane et al. 2006). The last two applications are beyond the scope of this review and, thus, will not be further considered.

Many factors influence the likelihood of successful transformation of the mother plant tissue and hairy root induction, including the species, age and type of plant tissue (Sevon and Oksman-Caldentey 2002), the type of *Agrobacterium* strain and the density of the bacterial suspension (Park and Facchini 2000). A number of chemicals may also promote these processes, e.g., acetosyringone (Fig. 1; Joubert et al. 2002). A new technique, named sonication-assisted *Agrobacterium*-mediated transformation (SAAT), to induce the formation of hairy roots in plant species that are difficult to transform, has also been

developed recently. Trick and Finer (1997) observed that the SAAT treatment produces small, uniform fissures and channels in tissues of various plants, which facilitate the access of *A. rhizogenes* to the internal plant tissues. Recently, this technique was also shown to be effective for transforming the hypocotyl of *Papaver* plants by Le Flem-Bonhomme et al. (2004).

Hairy roots—in vitro systems for producing valuable phytochemicals

The high biosynthetic potential of transformed root cultures was largely neglected for years, and the investigations that were performed on them mainly focused on the mechanisms underlying hairy root syndrome. However, during the mid-1980s and early 1990s, there were several investigations on their production of biologically active substances, especially alkaloids (Kamada et al. 1986; Flores et al. 1987), which revealed the biosynthetic capacity of the transformed root systems. Nowadays, hairy root cultures receive more attention as biological matrices for producing valuable metabolites, as they have several attractive features, including high genetic stability (compared to undifferentiated cultures) and relatively fast growth rates (compared to normal roots). Furthermore, growth regulators are not required for their cultivation, which is an important consideration, as some hormones are toxic (2,4-dichlorophenoxyacetic acid, for example), so their presence in many end products is unacceptable. In addition, use of hairy root systems is advantageous for the production of a number of secondary metabolites that are synthesized in plant roots and then accumulated in aerial parts of the plant (the naphthoquinone derivative lawsone, for example), as such metabolites are accumulated at very low amounts, or not at all, in undifferentiated or shoot cultures (Shanks and Morgan 1999). Table 1 lists some of the phytochemicals produced by hairy root cultures, according to reports published from 2000 to 2006.

One of the aspects of transformed root cultures that has attracted intense commercial interest is their potential for protein production (Shanks and Morgan 1999). The proteins they can produce include medicinally important enzymes such as superoxide dismutase (EC 1.15.1.1; Hyeon and Yoo 2002) and peroxidase (EC 1.11.1.7; Kim and Yoo 1996), other enzymes such as phytase (EC 3.1.3.8; using genetically engineered sesame hairy roots; Jin et al. 2005) and foreign proteins, e.g., monoclonal antibodies (Sharp and Doran 2001) and ribosome-inactivating proteins (Thorup et al. 1994). Recently, Doran (2006) reviewed the current status and problems associated with the production of foreign proteins by hairy root cultures (such as low accumulation levels, instability in the culture medium, etc.) and outlined strategies to minimize their degradation and losses. Although some of these problems have not yet been fully resolved, there is little doubt that these applications will continue to expand in the future.

In addition to proteins and well-known, standard metabolites of the mother plant, hairy roots are also potential sources of new, natural compounds. For example, Li et al. (1998) isolated a new compound named licoagrodione from *Glycyrrhiza glabra* cultures, which was shown to possess strong antimicrobial activities, and Berkov et al. (2003) reported the biosynthesis of a new tropane alkaloid ester in tetraploid hairy roots of *Datura stramonium*. Thus, hairy root cultures offer possibilities for

Table 1 Examples of metabolites produced by transformed root cultures[a]

Metabolite	Hairy root culture	Biological activity	Reference
Ajmalicine, ajmaline	*Rauvolfia micrantha*	Antihypertensive	Sudha et al. 2003
Artemisinin	*Artemisia annua*	Antimalarial	Weathers et al. 2005
Benzylisoquinoline alkaloids	*Papaver somniferum*; *Eschscholzia californica*	Analgesic, antibiotic	Park and Facchini 2000
Betalains	*Beta vulgaris*	Antioxidant, colorant	Pavlov and Bley 2006
Camptothecin	*Ophiorrhiza pumila*; *Camptotheca acuminata*	Antitumor	Saito et al. 2001; Lorence et al. 2004
Iridoid glycosides	*Harpagophytum procumbens*	Anti-inflammatory; analgesic and antidiabetic	Georgiev et al. 2006a
3,4-Dihydroxy-L-phenylalanine	*Stizolobium hassjoo*	Therapeutic agent against Parkinson's disease	Sung and Huang 2006
Rutin, hispidulin and syringin	*Saussurea involucrata*	Anti-inflammatory, antifungal	Fu et al. 2006
Scopolamine and hyoscyamine	*Datura innoxia*	Anticholinergic	Dechaux and Boitel-Conti 2005
Taxol	*Taxus brevifolia*	Anticancer	Huang et al. 1997
Thiarubrine A	*Ambrosia artemisiifolia*	Antifungal, antibacterial, antiviral	Bhagwath and Hjortsø 2000
6-Methoxy-podophyllotoxin	*Linum album*; *Linum persicum*	Anticancer	Wink et al. 2005

[a] Additional examples can be found in recent reviews by Guillon et al. 2006a,b.

isolating and synthesizing new compounds with potential pharmaceutical value, and several screening programs have been initiated (Kwon et al. 1997).

Strategies for exploiting the secondary metabolism of transformed roots

In order for hairy roots-based technologies to be commercially viable, several criteria have to be met, one of which is to ensure that the yields of the products are acceptable. Because these yields are generally too low at present, several strategies for improving them are being developed, as illustrated in Fig. 3 and discussed below.

Selection Generally, the first step in any attempt to produce a substance from in vitro plant cultures is to select lines that produce as much as possible of it and to continue this selection process after any subsequent optimization steps. Due to the randomness regarding the site at which the T-DNA integrates into the host plant genome, the resulting hairy root cultures often show considerable variations in growth rates, product yields and accumulation patterns of secondary metabolites (Hu and Du 2006). Furthermore, although transformed root cultures are considered to be stable (Shanks and Morgan 1999), a certain amount of heterogeneity has been found in them, and repeated selection seems to be important for obtaining stable, high-productivity hairy root lines (Yukimune et al. 1994). An alternative way for selecting in vitro hairy root lines has been proposed by Flores and Medina-Bolivar (1995), involving the addition of hormones to induce dedifferentiation of hairy roots and subsequent generation of cell suspensions. In the cell suspension stage, well-established approaches for obtaining cell lines that produce high levels of targeted metabolites could be applied (Berlin 1990; Georgiev et al. 2006b), and stable hairy root systems could then be regenerated by simply removing the growth regulators from the medium.

Approaches based on *genetic engineering* represent a relatively new stream of developments. The expression of foreign genes encoding enzymes that catalyze key-limiting steps in the biosynthetic pathways leading to desired metabolites offers good opportunities for improving yields and/or the synthesis of new compounds. Such gene transfers could be comparatively easily performed, using the

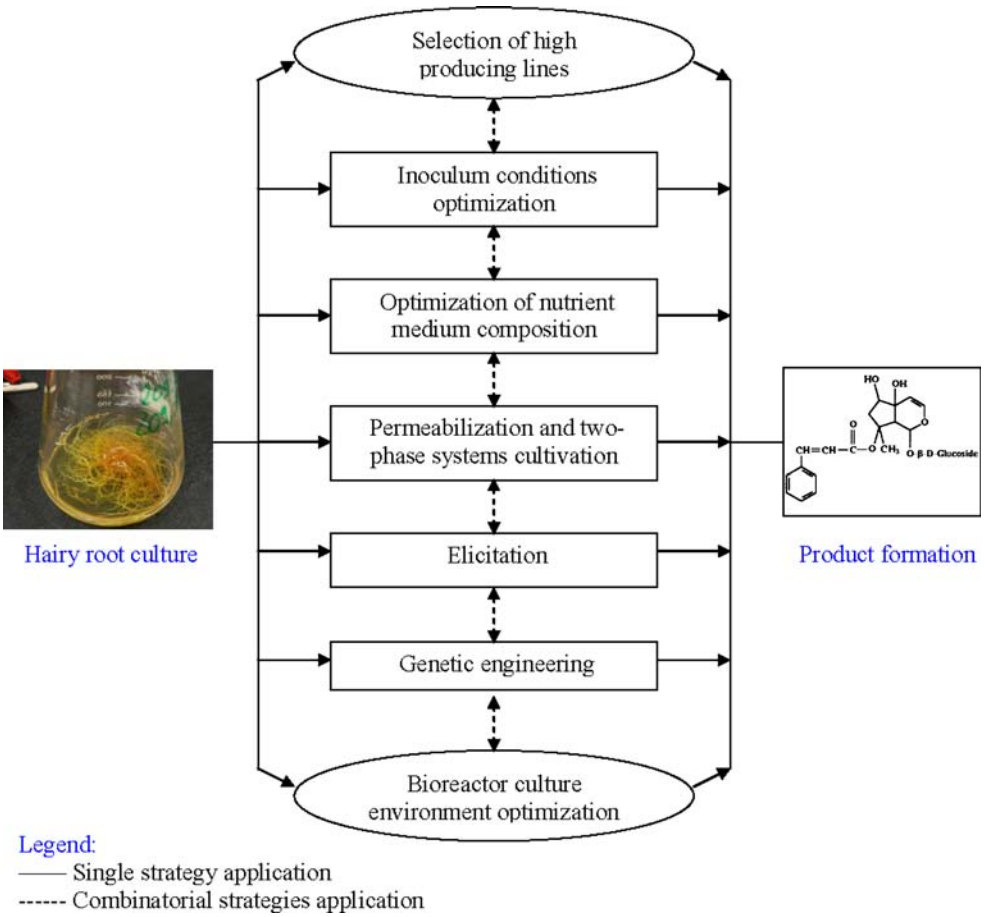

Fig. 3 Strategies for exploiting hairy root metabolism

natural genetic engineer (*A. rhizogenes*), by placing the desired gene(s) between the T-DNA borders and transferring the resulting construct into the host plant genome (Tzfira and Citovsky 2003). As examples of this approach, the gene encoding the enzyme hyoscyamine 6β-hydroxylase (EC 1.14.11.11), which catalyzes the conversion of hyoscyamine to scopolamine, has been successfully introduced into *Hyoscyamus muticus* hairy roots (Jouhikainen et al. 1999) and *Duboisia* hybrid hairy roots. In the latter case, over-expressing plants were also regenerated (Palazon et al. 2003a), and in both cited cases, the genetic manipulations resulted in enhanced scopolamine yields. In another more recent study, *Saussurea involucrate* was transformed with the chalcone isomerase gene from *Saussureamedusa*, and the transformed roots accumulated 12 times more apigenin and four times more total flavonoids than the wild-type roots (Li et al. 2006). However, progress in this field is still slow, largely because our knowledge of plant secondary metabolite pathways is limited (Verpoorte et al. 2002). A possible solution to this obstacle is suggested in a recently published work by Rischer et al. (2006), describing attempts to combine genome-wide transcript profiles (using complementary DNA–amplification fragment length polymorphism analyses) with metabolic profiles of elicited *Catharanthus roseus* cell suspensions. Although substantial further development is required, this kind of gene-to-metabolite network analysis seems to be quite promising.

Manipulating the chromosome numbers (*ploidy manipulation*) of in vitro hairy root systems may also provide effective means for both enhancing product yields and the biosynthesis of biologically active substances, which are not found in mother plants. Detailed information about the relationships between ploidy levels and secondary metabolite productivity can be found in Lavania (2005).

Optimization of the inoculum conditions The importance of optimizing the inoculum conditions is frequently underestimated. However, recent investigations have shown that inoculum size and age strongly influence the growth of *Panax ginseng* hairy root cultures (Jeong et al. 2004) and yields of betalains from *Beta vulgaris* hairy root cultures (Pavlov et al. 2003). The first group of cited authors found the growth rate of *P. ginseng* hairy roots to be maximal when a 0.7% (*w*/*v*) was used and significantly lower when a 0.4% (*w*/*v*) inoculum was used, and the optimal duration of subculture cycle was found to be 10 days (for *P. ginseng*) and 14 days for hairy roots of *B. vulgaris* (Pavlov et al. 2003; Jeong et al. 2004).

Optimization of nutrient medium composition The nutrient medium also affects hairy roots. Usually, the medium composition is modified with respect to its concentration (and ratios) of carbon, nitrogen and phosphorous sources (Wilhelmson et al. 2006) and other macronutrients (Sivakumar et al. 2005). A general approach for media optimization is to use statistical analysis (Sung and Huang 2000; Wilhelmson et al. 2006). In the first stage of this approach, the components of the nutrient media (independent variables) are varied, and the resulting culture growth and biosynthesis parameters of the desired metabolite(s) (process outputs) are determined. Multivariate analysis is then applied to explore the interactive "nutrient media–biomass–product" relationships between compounds in the biological systems. Depending on the relationships between the product biosynthesis and root growth parameters, the media can then be either optimized for both growth and production simultaneously or a two-stage (growth followed by bio-production) strategy can be developed.

Elicitation Elicitors are chemicals or biofactors from various sources that can induce physiological and morphological responses and phytoalexin accumulation (Zhao et al. 2005). Hence, elicitors are widely used tools for improving the yields of secondary metabolites that have defense functions in plant cells (Table 2). In recent years, a tendency to exploit abiotic elicitors has developed, as they are generally cheaper than biotic counterparts, and the preparation of some biotic elicitors requires additional facilities for cultivating the source microorganism(s). For example, vanadyl sulfate ($VOSO_4$), which has been found to be effective in several cases (Bhagwath and Hjortsø 2000; Palazon et al. 2003b), will enhance process costs by about 50 EUR (at concentration 50 mg/l) for production runs in a thousand-liter reactor.

Permeabilization and cultivation in two-phase systems Plant secondary metabolites are generally located in intracellular compartments, usually the vacuoles (Muehlbach 1998). Therefore, the production of many metabolites is limited by the capacity of the vacuole(s). To enhance yields of desired metabolites and reduce total process costs, several approaches for permeabilizing the cells and thereby releasing products have been applied, e.g., treatment with organic solvents (dimethyl sulfoxide; Sim et al. 1994) or surfactants (Boitel-Conti et al. 1996). For instance, treating *Datura innoxia* hairy roots with Tween 20 led to the migration of significant amounts of alkaloids from cells into the culture medium in a study by Boitel-Conti et al. (1996). Furthermore, the cited authors observed that total alkaloids production (intracellular plus extracellular) was 3- to 8-fold higher in treated than in untreated roots.

The addition of a second, artificial phase can also enhance the yields of target metabolites (Doernenburg and Knorr 1995). Removal of the product(s) by an artificial phase can be especially effective for increasing the total production of products that are subject to feedback

Table 2 Examples of secondary metabolite yield increases through elicitation[a]

Elicitor	Hairy root culture	Metabolite	Elicitor concentration	Fold increase	Reference
Abiotic elicitors					
$CdCl_2$	*Brugmansia candida*	Hyoscyamine	2 mM	1.35	Pitta-Alvarez et al. 2000
Jasmonic acid	*Panax ginseng*	Ginsenoside	5.0 mg/l	4.0	Sivakumar et al. 2005
Methyl jasmonate	*Panax ginseng*	Ginsenoside	22.4 mg/l	4.0	Palazon et al. 2003b
Vanadyl sulfate	*Ambrosia artemisiifolia*	Thiarubrine A	50 mg/l	8.0	Bhagwath and Hjortsø 2000
Biotic elicitors					
Enterobacter sakazaki	*Ammi majus*	Umbelliferone	15-ml Suspension/l	1.2	Staniszewska et al. 2003
Fusarium conglutinans	*Tagetes patula*	Total thiophenes	0.12 mg Carbohydrate/ml medium	2.7	Mukundan and Hjortsø 1990
Phytopthora parasitica (medium filtrate)	*Cichorium intybus*	Esculin and esculetin	0.1% (*v*/*v*)	4.1 and 3.7	Bais et al. 2000
S. aureus	*Scopolia parviflora*	Scopolamine	133.3 ml Suspension/l	2.8	Jung et al. 2003
Yeast elicitor	*Salvia miltiorrhiza*	Rosmarinic acid	–	2.3	Chen et al. 2001
Yeast extract	*Brugmansia candida*	Scopolamine and hyoscyamine	0.8 mg/ml	2.0	Pitta-Alvarez et al. 2000

[a] Additional examples can be found in recent reviews by Guillon et al. 2006a,b

inhibition or degradation by enzymes or other agents. Amberlite XAD resins are widely used as a second phase (Doernenburg and Knorr 1995), partly because many products can adsorb to them non-covalently and non-ionically, facilitating their subsequent elution. Sim et al. (1994) found such use of Amberlite XAD resins (in this case XAD 7) increased both biomass and the accumulation of indole alkaloids in hairy root cultures of *C. roseus*, e.g., catharanthine production was increased to 21.8 mg/l in the presence of the resin from 15.3 mg/l in its absence.

Bioreactors for hairy roots and large-scale cultivation

Bioreactor cultivations represent the final step in the development of techniques for producing metabolites from plant in vitro systems (Bourgaud et al. 2001). Many types of bioreactors have been successfully used for cultivating transformed root cultures (inter alia conventional stirred tanks, stirred tanks with a separate impeller, bubble columns, mist reactors and balloon-type reactors; Curtis 2000). Thus, it would be difficult, if not impossible, to select the "best" bioreactor design for cultivating transformed roots. However, for successful scale-up of hairy root-based processes, whatever type of bioreactor is used, several factors should be considered, including the physiology, morphology, unusual rheological properties and high stress sensitivity of hairy roots (Wysokinska and Chmiel 1997). However, the bioreactor cultivation of hairy roots has been considered in several reviews (Wysokinska and Chmiel 1997; Curtis 2000; Kim et al. 2002). Therefore, only recent advances (post-2002) will be considered here. One such advance is the development of disposable wave bioreactor systems. The working principle of these systems is based on wave-induced agitation, which significantly reduces stress levels. Moreover, utilization of plastic disposable chambers (called Cellbags) minimizes the need for labor- and time-consuming cleaning and sterilization procedures and facilitates fulfillment of Good Manufacture Practice requirements (Eibl and Eibl 2006). The performance and ginsenoside production of *P. ginseng* hairy roots in 2-l wave bioreactors have been studied in detail (Palazon et al. 2003c). The results showed that both biomass accumulation and ginsenoside production were significantly higher in 2-l wave systems than in shaken flasks. Large-scale wave systems with capacities up to 600 l are now commercially available (source: Wave Biotech AG®, Tagelswangen, Switzerland).

Temporary immersion systems (RITA®, Vitpropic, Saint-Mathieu-de-Tréviers, France) have also been used, in our laboratories, for cultivating hairy root cultures from *B. vulgaris* (Pavlov and Bley 2005, 2006) and *Harpagophytum procumbens* (Georgiev unpublished results). Although the RITA® systems have been developed for plant in vitro propagation, their advantages (reduced hyperhydricity and lower consumable and labor costs; McAlister et al. 2005) make them attractive for hairy root cultivation. These systems also seem to be able to solve a problem (so-called hyperhydration), that is associated with the bioreactor cultivation of transformed root cultures in some cases (Georgiev unpublished results). The scale of the RITA® systems is about 200 ml, and the daily cumulative duration of the immersion (flooding) stage can vary from a minute to

several hours. Pavlov and Bley (2006) found that growth of *B. vulgaris* hairy roots is optimal with 15-min immersion/75-min standby cycles, while maximal amounts of betalain pigments are accumulated with 15-min flooding/60-min standby cycles.

Another significant problem that can arise during the cultivation of hairy roots in bioreactor is associated with mass transfer limitations and heterogeneities in root oxygen demands, which are greater in the meristems than the mature tissue (Asplund and Curtis 2001). The hairy roots typically grow in a "tuft-like" manner, which promotes the formation of oxygen and nutrient gradients in the tissue. Curtis (2000) concluded that at low tissue concentrations (<10-g dry weight/l), hairy roots can be grown in bioreactors with virtually any configuration. However, at high tissue concentrations (>10-g dry weight/l) in submerged bioreactors several scale-up limitations may arise. For high tissue density cultivation, Ramakrishnan and Curtis (2004) developed a 14-l pilot-scale reactor that operates in a "trickle-bed" mode. The dry mass of *H. muticus* cultivated in this bioreactor reached 36 g/l within 25 days, and the calculated growth index (based on the dry weight) and doubling time were 180 and 3.3 days, respectively, the fastest growth parameters reported for hairy roots so far. In addition, an attempt to cultivate transformed root cultures on a large-scale (500 l) has been reported by Wilson (1997), using a procedure involving inoculum preparation in a 10-l seed vessel for 2 weeks, followed by aseptic transfer of the root inoculum to the main 500-l reactor, where the roots were immobilized on barbs, which further facilitate harvesting of the root mass. Although the final biomasses yield (about 4-g dry weight/l) could not be considered high, this procedure addresses many potentially problematic issues (mainly technological) concerned with transferring the tissue inoculum from the seed reactor to the main one(s).

Monitoring the roots' growth during the processes in reactor systems is highly important. Unlike in plant cell suspension-based processes, during the cultivation of organ cultures (e.g. hairy roots), it is impossible to obtain homogeneous samples of the tissue, which complicates measurements of growth and other process parameters. Therefore, conductivity measurements have been widely used to obtain indirect growth estimates (Heyon and Yoo 2003; Pavlov and Bley 2006). Changes in conductivity are due to the cellular uptake of ionic nutrients (NO_3^- anions usually). Linear relationships between increases in biomass and reductions in medium conductivity can be expressed with the equation:

$$(X_f - X_i) = (C_i - C_f)\beta, \quad (1)$$

where X_f and X_i are the final and initial biomass (g), respectively; C_f and C_i are the final and initial medium conductivity (mS/cm), respectively; and β (g cm/mS) is a proportional coefficient. However, the cells in the cultures pass through different growth phases (in which rates of uptake of the nutrients vary), and thus, Eq. (1) can only be strictly applied for exact measurements during the exponential growth phase. It is worth noting that, in some cases, such linear relationships between reductions in conductivity and biomass growth increase do not exist (Georgiev et al. 2006a), indicating that the relationships should be individually determined for every culture. The osmolarity of the culture medium offers another possibility for indirect measurements of the root growth. Osmolarity measurements have several advantages over conductivity measurements, as osmolarity takes into account the total number of moles of all solutes present in the medium (Suresh et al. 2001).

Finally, Lee et al. (1998) observed correlations between redox potential (*Eh*) and glucose, cell and ornithine concentrations and proposed that this parameter could be applied for online measurements of *Brevibacterium ketoglutamicum* growth parameters and metabolite biosynthesis. Use of such indicators could also be considered for monitoring hairy roots growth. Huang and Chou (2006) found that redox potential changes during the cultivation of hairy root cultures in a mist trickling reactor reflect the assimilation of NH_4^+, NO_3^-, sucrose and growth phases.

Future prospects

Recent advances in *Agrobacterium*-mediated transformation techniques allow hairy root systems to be induced from large numbers of plant species, including rare and threatened medicinal plants, which should contribute significantly to future attempts to preserve global biodiversity and alleviate associated ecological problems. The immense potential of transformed root cultures for biosynthesizing valuable metabolites has been recognized. Furthermore, the advantages of hairy roots make them attractive alternatives to classical technologies and even plant cell suspension cultures. Further enhancement of knowledge regarding plant metabolic pathways and the mechanisms of their regulation in the near future should give us powerful tools for exploiting more fully the biosynthetic potential of hairy roots and for designing new ways to synthesize compounds. All of this suggests that hairy roots technologies are on the threshold of a new era, in which industrial scale bioproduction will be achieved. Of course, several problems must be addressed, which without any doubt will be solved through deeper collaboration between specialists from various relevant fields.

Acknowledgements We thank V. Georgiev, M.Sc., for preparing the figures. This work has been supported by a Marie Curie Fellowship of the European Community program "Development Host Fellowships"

under contract number HPMD-CT-2001-00092 and Marie Curie European Reintegration Grant under contract number MERG-CT-2005-021344.

References

Asplund TA, Curtis WR (2001) Intrinsic oxygen use kinetics of transformed root culture. Biotechnol Prog 17:481–489

Bais HP, Govindaswamy S, Ravishankar GA (2000) Enhancement of growth and coumarin production in hairy root cultures of Witloof chicory (*Cichorium intybus* L. cv. Lucknow local) under the influence of fungal elicitors. J Biosci Bioeng 90:648–653

Berkov S, Pavlov A, Kovacheva P, Stanimirova P, Philipov S (2003) Alkaloid spectrum in diploid and tetraploid hairy root cultures of *Datura stramonium*. Z Naturforsch C 58:42–46

Berlin J (1990) Screening and selection for variant cell lines with increased levels of secondary metabolites. In: Charlwood BV, Rhodes MJC (eds) Secondary products from plant tissue culture. Clarendon, Oxford, pp 119–138

Bhagwath SG, Hjortsø MA (2000) Statistical analysis of elicitation strategies for thiarubrine A production in hairy root cultures of *Ambrosia artemisiifolia*. J Biotechnol 80:159–167

Boitel-Conti M, Gontier E, Laberche J-C, Ducrocq C, Sangwan-Noreel BS (1996) Inducer effect of Tween 20 permeabilization treatment used for release of stored tropane alkaloids in *Datura innoxia* Mill. hairy root cultures. Plant Cell Rep 16:241–244

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: a historical perspective. Plant Sci 161:839–851

Chen H, Chen F, Chiu FCK, Lo CMY (2001) The effect of yeast elicitor on the growth and secondary metabolism of hairy root cultures of *Salvia miltiorrhiza*. Enzyme Microb Technol 28:100–105

Chilton MD, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorogenesis. Cell 11:263–271

Chilton MD, Saiki RK, Yadav N, Gordon MP, Quetier F (1980) T-DNA from *Agrobacterium* Ti plasmid is in the nuclear DNA of crown gall tumour cells. Proc Natl Acad Sci USA 77:4060–4064

Choi PS, Kim YD, Chung HJ, Choi DW, Liu JR (2004) Plant regeneration from hairy-root cultures transformed by infection with *Agrobacterium rhizogenes* in *Catharanthus roseus*. Plant Cell Rep 22:828–831

Crane C, Wright E, Dixon RA, Wang Z-Y (2006) Transgenic *Medicago truncatula* plants obtained from *Agrobacterium tumefaciens*-transformed root and *Agrobacterium rhizogenes*-transformed hairy roots. Planta 223:1344–1354

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

Dechaux C, Boitel-Conti M (2005) A strategy for overaccumulation of scopolamine in *Datura innoxia* hairy root culture. Acta Biol Cracov Ser Bot 47:101–107

Doernenburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme Microb Technol 17:674–684

Doran PM (2006) Foreign protein degradation and instability in plants and plant tissue cultures. Trends Biotechnol 24:426–432

Eibl R, Eibl D (2006) Design and use of the wave bioreactor for plant cell culture. In: Gupta SD, Ibaraki Y (eds) Plant tissue culture engineering. Focus on biotechnology, vol 6. Springer, pp 203–227

Flores H, Medina-Bolivar H (1995) Root cultures and plant natural products: "unearthing" the hidden half of plant metabolism. In: Watad A (ed) Plant tissue culture and biotechnology, vol 1. Balaban Publisher, UK, pp 59–74

Flores HE, Hoy MW, Pickard JJ (1987) Secondary metabolites from root cultures. Trends Biotechnol 5:64–69

Fowler MW (2006) Plants, medicines and man. J Sci Food Agric 86 (12):1797–1804

Fu C-X, Xu Y-J, Zhao D-X, Ma FS (2006) A comparison between hairy root cultures and wild plants of *Saussurea involucrata* in phenylpropanoids production. Plant Cell Rep 24:750–754

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and interaction. Annu Rev Plant Physiol Plant Mol Biol 51:223–256

Georgiev M, Heinrich M, Kerns G, Pavlov A, Bley T (2006a) Production of iridoids and phenolics by transformed *Harpagophytum procumbens* root cultures. Eng Life Sci 6:593–596

Georgiev M, Pavlov A, Ilieva M (2006b) Selection of high rosmarinic acid producing *Lavandula vera* MM cell lines. Process Biochem 41:2068–2071

Guillon S, Tremouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006a) Hairy root research: recent scenario and exciting prospects. Curr Opin Plant Biol 9:341–346

Guillon S, Tremouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006b) Harnessing the potential of hairy roots: dawn of a new era. Trends Biotechnol 24:403–409

Heyon KJI, Yoo YJE (2002) Optimization of SOD biosynthesis by controlling sucrose concentration in the culture of carrot hairy root. J Microbiol Biotechnol 12:617–621

Heyon KJI, Yoo YJE (2003) Adaptive estimation of hairy root mass using conductometry. J Microbiol Biotechnol 13:641–646

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

Huang S-Y, Chou S-N (2006) Elucidation of the effects of nitrogen source on proliferation transformed hairy roots and secondary metabolite productivity in a mist trickling reactor by redox potential measurement. Enzyme Microb Technol 38:803–813

Huang Z, Mu Y, Zhou Y, Chen W, Xu K, Yu Z, Bian Y, Yang Q (1997) Transformation of *Taxus brevifolia* by *Agrobacterium rhizogenes* and taxol production in hairy root culture. Acta Bot Yunnanica 19:292–296

Jeong G-T, Park D-H, Ryu H-W, Hwang B, Woo J-C (2004) Effects of inoculum conditions on growth of hairy roots of *Panax ginseng* C.A. Meyer. Appl Biochem Biotechnol 113–116:1193–1203

Jin UH, Chun JA, Han MO, Lee JW, Yi YB, Lee SW, Chung CH (2005) Sesame hairy root cultures for extra-cellular production of a recombinant fungal phytase. Process Biochem 40:3754–3762

Joubert P, Beaupere D, Lelievre P, Wadouachi A, Sangwan RS, Sangwan-Norreel BS (2002) Effect of phenolic compounds on *Agrobacterium vir* genes and gene transfer induction—a plausible molecular mechanism of phenol binding protein activation. Plant Sci 162:733–743

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey KM (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551

Jung H-Y, Kang S-M, Kang Y-M, Kang M-J, Yun D-J, Bahk J-D, Yang J-K, Choi M-S (2003) Enhanced production of scopolamine by bacterial elicitors in adventitious hairy root cultures of *Scopolia parviflora*. Enzyme Microb Technol 33:987–990

Kamada H, Okamura N, Satake M, Harada H, Shimomura K (1986) Alkaloid production by hairy root cultures in *Atropa belladonna*. Plant Cell Rep 5:239–242

Kieran P (2001) Bioreactor design for plant cell suspension cultures. In: Cabral JMS (ed) Principles of multiphase reactor design. Harwood Academic Publishers, pp 391–426

Kim YH, Yoo YJ (1996) Peroxidase production from carrot hairy root cell culture. Enzyme Microb Technol 18:531–535

Kim Y, Wyslouzil BE, Weathers PJ (2002) Secondary metabolism of hairy root cultures in bioreactors. In Vitro Cell Dev Biol Plant 38:1–10

Kwon BM, Ro SH, Kim MK, Nam JY, Jung HJ, Lee IR, Kim YK, Bok SH (1997) Polyacetilene analogs isolated from hairy roots of *Panax ginseng*, inhibit acyl-CoA-cholesterol. Planta Med 63:552–553

Lavania U (2005) Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. Plant Gene Res 3:170–177

Le Flem-Bonhomme V, Laurain-Mattar D, Fliniaux MA (2004) Hairy root induction of *Papaver somniferum* var. *album*, a difficult-to-transform plant by *A. rhizogenes* LBA 9402. Planta 218:890–893

Lee TH, Chang YK, Chung BH, Park YH (1998) Correlation of redox potential with state variables in cultures under controlled dissolved oxygen concentration and pH. Biotechnol Prog 14:959–962

Li W, Asada Y, Yoshikawa T (1998) Antimicrobial flavonoids from *Glycyrrhiza glabra* hairy root cultures. Planta Med 64:746–747

Li F-X, Jin Z-P, Zhao D-X, Cheng L-Q, Fu C-X, Ma F (2006) Overexpression of the *Saussurea medusa* chalcone isomerase gene in *S. involucrate* hairy root cultures enhances their biosynthesis of apigenin. Phytochemistry 67:553–560

Lorence A, Medina-Bolivar F, Nessler CL (2004) Camptothecin and 10-hydroxycamptothecin from *Camptotheca acuminata* hairy roots. Plant Cell Rep 22:437–441

Mano Y, Nabeshima S, Matsui C, Ohkawa H (1986) Production of tropane alkaloids by hairy root cultures of *Scopolia japonica*. Agric Biol Chem 50:2715–2722

McAlister B, Finnie J, Watt MP, Blakeway F (2005) Use of the temporary immersion bioreactor system (RITA®) for the production of commercial *Eucalyptus* clones in Mondi Forests (SA). Plant Cell Tissue Organ Cult 81:347–358

Muehlbach H-P (1998) Use of plant cultures in biotechnology. In: El-Gewely MR (ed) Biotechnology annual review, vol 4. Elsevier Science BV, pp 113–176

Mukundan U, Hjortsø MA (1990) Effect of fungal elicitor on thiophene production in hairy root cultures of *Tagetes patula*. Appl Microbiol Biotechnol 33:145–147

Nilsson O, Olsson O (1997) Getting to the root: the role of the *Agrobacterium rhizogenesrol*-genes in the formation of hairy roots. Physiol Plantarum 100:463–473

Nin S, Bennici A, Roselli G, Mariotti D, Schiff S, Magherini R (1997) *Agrobacterium*-mediated transformation of *Artemisia absinthium* L. (wormwood) and production of secondary metabolites. Plant Cell Rep 16:725–730

Palazon J, Moyano E, Cusido RM, Bonfill M, Oksman-Caldentey K-M, Pinol MT (2003a) Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the *h6h* gene. Plant Sci 165:1289–1295

Palazon J, Cusido RM, Bonfill M, Mallol A, Moyano E, Morales C, Pinol MT (2003b) Elicitation of different *Panax ginseng* transformed root phenotypes for an improved ginsenoside production. Plant Physiol Biochem 41:1019–1025

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

Park S-U, Facchini PJ (2000) *Agrobacterium rhizogenes*-mediated transformation of opium poppy, *Papaver somniferum* L., and California poppy, *Eschscholzia californica* Cham., root cultures. J Exp Bot 347:1005–1016

Pavlov A, Bley T (2005) Betalains biosynthesis by *Beta vulgaris* L. hairy root culture in different bioreactor systems. Scientific Works, University of Food Technologies 52:299–304

Pavlov A, Bley T (2006) Betalains biosynthesis by *Beta vulgaris* L. hairy root culture in a temporary immersion cultivation system. Process Biochem 41:848–852

Pavlov A, Kovatcheva P, Georgiev V, Koleva I, Ilieva M (2002) Biosynthesis and radical scavenging activity of betalains during the cultivation of Red beet (*Beta vulgaris*) hairy root cultures. Z Naturforsch C 57:640–644

Pavlov A, Georgiev V, Kovatcheva P (2003) Relationship between type and age of inoculum and betalains biosynthesis by *B. vulgaris* hairy root culture. Biotechnol Lett 25:307–309

Payne J, Hamill JD, Robins RJ, Rhodes MJC (1987) Production of hyoscyamine by hairy root cultures of *Datura stramonium*. Planta Med 53:474–478

Pitta-Alvarez SI, Spollansky TC, Guilietti AM (2000) The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Enzyme Microb Technol 26:252–258

Rahman L, Ikenaga T, Kitamura Y (2004) Penicillin derivatives induce chemical structure-dependent root development, and application for plant transformation. Plant Cell Rep 22:668–677

Ramakrishnan D, Curtis WR (2004) Trickle-bed root culture bioreactor design and scale-up: growth, fluid-dynamics and oxygen mass transfer. Biotechnol Bioeng 88:248–260

Rao SR, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Morena DA, Ripoll C, Yakoby N, O'Neal J, Cornwell T, Pastor I, Fridlender B (2002) Plants and human health in the twenty-first century. Trends Biotechnol 20:522–531

Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MCE, Inze D, Oksman-Caldentey K-M, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA 103:5614–5619

Saito K, Sudo H, Yamazaki M, Koseki-Nakamura M, Kitajima M, Takayama H, Aimi N (2001) Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila*. Plant Cell Rep 20:267–271

Sevon N, Oksman-Caldentey K-M (2002) *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. Planta Med 68:859–868

Shanks JV, Morgan J (1999) Plant "hairy root" culture. Curr Opin Biotechnol 10:151–155

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

Sim SJ, Chang HN, Liu JR, Jung KH (1994) Production and secretion of indole alkaloids in hairy root cultures of *Catharanthus roseus*: effect of in situ adsorption, fungal elicitation and permeabilization. J Ferment Bioeng 78:229–234

Sivakumar G, Yu KW, Hahn EJ, Paek KY (2005) Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors. Curr Sci 89:641–649

Staniszewska I, Krolicka A, Malinski E, Lojkowska E, Szafranek J (2003) Elicitation of secondary metabolites in in vitro cultures of *Ammi majus* L. Enzyme Microb Technol 33:565–568

Sudha CG, Obul Reddy B, Ravishankar GA, Seeni S (2003) Production of ajmalicine and ajmaline in hairy root cultures of *Rauvolfia micrantha* Hook f., a rare and endemic medicinal plant. Biotechnol Lett 25:631–636

Sung L-S, Huang S-Y (2000) Medium optimization of transformed root cultures of *Stizolobium hassjoo* producing L-DOPA with response surface methodology. Biotechnol Prog 16:1135–1140

Sung L-S, Huang S-Y (2006) Lateral root bridging as a strategy to enhance L-DOPA production in *Stizolobium hassjoo* hairy root cultures by using a mesh hindrance mist trickling bioreactor. Biotechnol Bioeng 94:441–447

Suresh B, Rajasekaran T, Rao SR, Raghavarao KSMS, Ravishankar GA (2001) Studies on osmolarity, conductivity and mass transfer

for selection of a bioreactor for *Tagetes patula* L. hairy roots. Process Biochem 36:987–993

Thorup JE, McDonald KA, Jackman AP, Bhatia N, Dandekar AM (1994) Ribosome-inactivating protein production from *Trichosanthes kirilowii* plant cell cultures. Biotechnol Prog 10:345–352

Trick HN, Finer JJ (1997) SAAT: sonication-assisted *Agrobacterium*-mediated transformation. Transgenic Res 6:329–336

Tzfira T, Citovsky V (2003) The *Agrobacterium*–plant cell interaction. Taking biology lessons from a bug. Plant Physiol 133:943–947

Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet 20:375–383

Uozumi N (2004) Large-scale production of hairy root. In: Scheper T, Kobayashi T (eds) Advances in biochemical engineering/biotechnology, vol 91. Springer, Berlin, pp 75–103

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1:13–25

Weathers P, Bunk G, McCoy MC (2005) The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. In Vitro Cell Dev Biol Plant 41:47–53

Weising K, Kahl G (1996) Natural genetic engineering of plant cells: the molecular biology of crown gall and hairy root disease. World J Microbiol Biotechnol 2:327–351

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

Wilson PDG (1997) The pilot-scale cultivation of transformed roots. In: Doran PM (ed) Hairy roots and culture applications. Harwood Academic Publishers, 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 bioproduction of phytochemicals by plant in vitro cultures: anticancer agents. Plant Gene Res 3:90–100

Wysokinska H, Chmiel A (1997) Transformed root cultures for biotechnology. Acta Biotechnol 17:131–159

Xie D, Zou Z, Ye H, Li H, Guo Z (2001) Selection of hairy root clones of *Artemisia annua* L. for artemisinin production. Isr J Plant Sci 49:129–134

Yukimune Y, Hara Y, Yamada Y (1994) Tropane alkaloid production in root cultures of *Duboisia myoporoides* obtained by repeated selection. Biosci Biotechnol Biochem 58:1443–1446

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333