



HAL
open science

Induction and growth of hairy roots for the production of medicinal compounds

Lamine Bensaddek, Maria Luisa Villarreal, Marc-André Fliniaux

► **To cite this version:**

Lamine Bensaddek, Maria Luisa Villarreal, Marc-André Fliniaux. Induction and growth of hairy roots for the production of medicinal compounds. *Electronic Journal of Integrative Biosciences*, 2008, 3 (1, Special Issue on Hairy Roots (A. Lorence and F. Medina-Bolivar, co-editors)), pp.2-9. <hal-00602520>

HAL Id: hal-00602520

<https://hal.science/hal-00602520v1>

Submitted on 23 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Induction and growth of hairy roots for the production of medicinal compounds

L Bensaddek¹, ML Villarreal², MA Fliniaux¹

¹ Laboratoire de Phytotechnologie (EA 3900) Université de Picardie Jules Verne, 80000 Amiens, France

² Centro de Investigacion en Biotecnologia, Universidad Autonoma del Estado de Morelos (UAEM), CP 62210 Cuernavaca, Morelos, Mexico

Abstract

The development of genetically transformed plant tissue cultures and mainly of roots transformed by *Agrobacterium rhizogenes* (hairy roots), is a key step in the use of *in vitro* cultures for the production of secondary metabolites. Hairy roots are able to grow fast without phytohormones, and to produce the metabolites of the mother plant. The conditions of transformation (nature and age of the explants, bacterial strain, bacterial density, and the protocol of infection) deeply influence the frequency of the transformation events as well as the growth and productivity of the hairy roots. Then optimization of the culture parameters (medium constituents, elicitation by biotic or abiotic stress) may enhance the capability of the hairy roots to grow fast and to produce valuable compounds.

Introduction

The use of plants as medicines is a very ancient story and a traditional medical practice in all the passed civilisations (Samuelsson, 2004). Natural products and in particular plant metabolites are still extensively used for therapeutic applications, and it was evaluated that between 1981 and 2002, 28% of the 868 new chemical entities were natural products or derived from natural products, with another 24% created around a pharmacophore from a natural product (Raskin et al., 2002; Newman et al., 2003; Balunas and Kinghorn, 2005). In the early years of the twenty-first century, plants are economically important pharmaceuticals and essential for human health. Examples of important drugs obtained from plants are, morphine and codeine from *Papaver somniferum*, vincristine and vinblastine from *Catharanthus roseus*, digoxin from *Digitalis lanata* (Hollman, 1996), and quinine and quinidine from *Cinchona* spp. Natural products have played a major role in lead discovery, mainly in the following areas: oncology, cardiovascular and metabolic diseases, and immuno suppression (Butler, 2004). It has been estimated that between 1981 and 2002, 60% of anti-cancer drugs and 75% of anti-infectious drugs already on the market or under clinical trial were of natural origin (Cragg et al., 1997; Yue-Zhong Shu, 1998; Newman et al., 2003; Lam, 2007). The antimalarial artemisinin, isolated from *Artemisia annua* L. is effective against multidrug resistant strains of *Plasmodium*, and a lead compound for the discovery of new antimalarial drugs (van Agtmael et al., 1999). Several clinically useful anti-cancer agents are plant products or their close derivatives: vinblastine, irinotecan, topotecan, etoposide, and paclitaxel (Cragg and Newman, 2005). Huperzine A and galantamine (galanthamine) acting as acetylcholinesterase inhibitors have been approved for the treatment of Alzheimer's disease and other neurodegenerative pathologies (Raves et al., 1997; Scott and Goa, 2000). The obtention of medicinal compounds from extraction of wild or cultivated plants can be limited by various problems: plants difficult to cultivate, risk of extinction for over exploited plants, and geopolitical problems, among other causes (Verpoorte et al., 2002).

To try to overcome these problems, many attempts were made during the last decades to evaluate the possibility of producing medicinal compounds by *in vitro* plant cell and organ cultures (Berlin, 1986; Alfermann and Petersen 1995). However, in most cases, the compounds were undetectable or were accumulated at low levels in the cultures. Several strategies such as screening and selection of high producing cell lines, cell immobilization, elicitation, and culture of differentiated tissues were developed. In each case problems were

encountered and results did not allow the development of an economically valuable commercialization of the biotechnologically produced compounds (Verpoorte et al., 2002).

The *in vitro* transformation of plant material with *Agrobacterium rhizogenes* strains allowed to overcome some of the huge difficulties of *in vitro* plant organ cultures, and led to the obtention of fast growing organs, exhibiting extensive branching, and capable of producing the main metabolites of the mother plant or even new metabolites undetected in the mother plant nor in other kinds of *in vitro* cultures (Nader et al., 2006). The so called hairy roots offered a promising technology for secondary metabolite production (Hamill et al., 1987) such as tropane alkaloids (Flores and Filner 1985; Oksman-Caldentey and Arroo, 2000) and many other metabolites (Giri and Narasu, 2000).

At the present time, the more precise knowledge about *A. rhizogenes* transformation of plant material as well as about hairy roots and their biotechnological use for the production of pharmaceutical products offer new prospects (Guillon et al., 2006a and b; Kuzovkina and Schneider, 2006; Georgiev et al., 2007; Srivastava and Srivastava, 2007).

***Agrobacterium rhizogenes* strains and the induction of hairy roots**

In the hairy roots disease, the infectious process by *A. rhizogenes* wild strains is characterized by the following four steps: 1) chemotactism induced movement of agrobacteria towards the plant cells; 2) binding of the bacteria to the surface components of the cell wall; 3) activation of the virulence (*vir*) genes, and 4) transfer and integration of the transfer-DNA (T-DNA) into the plant genome (Zupan and Zambryski, 1997). The genetic information allowing this infection process is mainly contained in the Ri plasmid (pRi) carried by the bacteria. In the pRi, the *vir* region concentrates 6 to 8 genes involved in the DNA transfer. The right and left T-DNA regions (T_R-DNA and T_L-DNA) of the pRi, which are delimited by their border sequences, are the regions that are transferred to the plant.

Within the T_R section, loci involved in auxin biosynthesis are transferred to the plant genome, thus increasing the auxin level of the transformants. Other genes of the T_R section are responsible of the synthesis of opines which are unusual amino acid sugar derivatives used by the bacteria for their feeding (Gartland, 1995).

The wild *A. rhizogenes* strains, many of which have been used to produce hairy roots from medicinal plants, can be classified by their opine type. Agropine strains (A4, 15834, 1855, LBA 9402) induce agropine, mannopine and agropinic acid production while the mannopine strains (8196) and the cucumopine (Petit et al., 1983) strains induce the production of one single opine. Agropine strains pRi transfer independently both the T_L-DNA and T_R-DNA to the plant genome, while mannopine strains only transfer the T_L-DNA. This pRi region contains the four *rol* genes A, B, C and D (Schmülling et al., 1988; Petersen et al., 1989) which enhance the auxin and cytokinin (Estruch et al., 1991) susceptibility of plant cells and are responsible for the formation of roots by transformed tissues (Bonhomme et al., 2000a; Hong et al., 2006). The hairy root phenotype is mainly due to the *rol* genes (A, B, C and D), and in particular the *rol B* gene (Nilsson and Olsson, 1997), though hairy roots could also be obtained after transformation of *Atropa belladonna*, with the *rol C* gene alone (Bonhomme et al., 2000b). The choice of a bacterial strain is very important since some plants are very resistant to infection (monocots are for example harder to transform with *Agrobacterium* than dicotyledonous plants). Moreover, bacterial strains are more or less virulent according to the plant species. The LBA 9402 strain is hypervirulent and has been used to successfully transform *Hyoscyamus muticus* (Vanhala et al., 1995), *Centaureum erythraea* (Piatczak et al., 2006), *Saponaria vaccaria* (Schmidt et al., 2007), *Gentiana macrophylla* (Tiwari et al., 2007) and *Papaver somniferum album* (Le Flem et al., 2004). Besides the use of wild strains, genetically engineered bacterial strains with modified pRi or disarmed *Agrobacterium tumefaciens* with a plasmid containing *rol* genes together or separately have been also employed for the transformation. Hairy roots may also be initiated, containing constitutive expression constructs. This was the case for *Cinchona officinalis* (Geerlings et al., 1999). These authors developed a binary vector whose T-DNA contained constitutive-expression

versions (CaMV35S promoter with double enhancer and *nos* terminator) of two genes encoding rate-limiting enzymes: tryptophan decarboxylase (*tdc*) and strictosidine synthase (*str*) from *C. roseus*, together with an intron-possessing β -glucuronidase (*gus-int*) reporter gene and a hygromycin phosphotransferase (*hpt*) selection marker gene. This binary vector construct was used in conjunction with *A. rhizogenes* strain LBA 9402 to obtain *tdc* and *str*-gene-transformed hairy roots of *C. officinalis*. This technique opened a wide field of applications in the regulation of biosynthetic pathways and bioconversions; however, plant transgenesis is still a discussed subject which encounters a strong opposition of the public opinion, more specifically in European countries.

Infection conditions of the plant material

Several protocols have been used for the infection of plant material by *A. rhizogenes*. However, the success of the transformation depends on various parameters such as the species and the age of the plant tissue, with the younger ones being in general more sensitive to bacterial infection (Sevon and Oksman-Caldentey, 2002). The bacterial strain used and the density of the bacterial suspension are also influential (Park and Facchini, 2000). The explants most commonly used for infection are young tissues of sterile plantlets, hypocotyl segments, cotyledons, petioles and young leaves. The contact between bacteria and plant cells can be induced by direct injection of the bacterial suspension into the plantlet or by immersion of the plant tissues in the bacterial suspension. This last procedure can be enhanced with vacuum infiltration (Tomilov et al., 2007). In these cases, the explants have to be wounded before they are inoculated. The use of excised tissues, leaf disks (Wang et al., 2002) or organ sections (Komaraiah et al., 2003) increases the contact surface between the plant tissue and the bacteria. With hard to transform plants alternative procedures may be implemented. Among these procedures, micro wounding through electroporation (Matsuki et al., 1989) or sonication can be used (in a process called sonication assisted *Agrobacterium*-mediated transformation or SAAT) (Trick and Finer, 1997; Le Flem et al., 2004). For the transformation of *P. somniferum album*, several factors of the SAAT protocol were investigated for their influence on transient *gus* expression: pre-culture period, sonication and co-culture duration. The highest number of GUS - positive hypocotyls (91%) was obtained after 60 seconds of sonication and 2 days of co-culture.

In several experiments acetosyringone was used to activate the virulence genes of *Agrobacterium*, and to enhance the transfer of foreign genes into the plant genome (Stachel et al., 1985; Gelvin, 2000; Tao and Li, 2006; Kumar et al., 2006). The optimal concentration of acetosyringone varies from one experiment to another. For the transformation of *Torenia fournieri*, low concentrations (10-30 μ M) enhanced the transformation, but higher ones did not significantly increase the transformation frequency. For *Nicotiana tabacum* or *P. somniferum* the acetosyringone concentrations used varied between 50 and 150 μ M.

The duration of the plant - bacterium contact during the inoculation and the co-cultivation are parameters that can be optimized. The average co-cultivation duration is about two or three days. After that, the explants must be transferred to a solid medium containing an antibiotic to eliminate the bacteria. Cefotaxime (250-500 mg L⁻¹) and Timentin (200-300 mg L⁻¹) are often used to eliminate the bacteria. The explants are then transferred onto a solid hormone-free medium in the dark at 20-25°C, and the first roots appear after a few weeks (usually 1 to 4). The roots are then transferred to erlenmeyer flasks containing liquid phytohormones -free medium. The typical transformed root phenotype is a highly branched root covered with a mass of tiny root hairs and these cultures do not require phytohormones. Concerning the growth rate, the average doubling time of hairy root lines is around 2-3 days. Species-related anatomic, morphologic and cytologic changes have been reported (Webb et al., 1990).

The putatively transformed roots are usually analyzed to check for T-DNA integration. Opine analysis using paper electrophoresis on root extracts is one of the techniques used to confirm the transgenic nature of the roots (Giri et al., 2000; Han et al., 2006). Another alternative is the use of reporter genes. In this area, the use of binary vectors has proven

useful for assessing the gene transfer to the plant genome, and following the long term stability of this transfer using selection or marker genes. Selection genes can be antibiotic – resistance genes such as *nptII* or *hpt* coding for neomycin phosphotransferase or hygromycin –phosphotransferase, respectively, expressed only in the transformed tissues. A study on *Astragalus sinicus* and *Glycine max* using feedback-insensitive anthranilate synthase (ASA2) cDNA isolated from a 5-methyltryptophan (5MT)-resistant tobacco cell line showed that hairy roots transformed with a *35s-asa2* construct could be directly selected using 20–75 μM 5MT. GUS staining or fluorescence microscopy following transformation with binary vectors containing GUS, GFP, dsRED or EYFP are useful for the identification of stable transformed roots (Tomilov et al., 2007). PCR and Southern-blotting of *rol* genes is also another way to confirm T-DNA integration into the plant genome (Lorence et al., 2004; Tiwari et al. 2007). Additional PCR and Southern-blotting of *vir C* gene is sometimes performed to check for total elimination of the agrobacteria (Shi et al., 2006).

The infection conditions are of capital importance and the choice of the *Agrobacterium* strain is a first - rate parameter. The strain virulence has strong repercussions on the transformed material properties (morphology, growth rate, and metabolite level). The *A. rhizogenes* strain LBA 9402 has showed stronger infective ability on *Rheum palmatum* while *A. rhizogenes* strain R1601 generated a faster growing clone. In this paper, like often reported, the secondary metabolite content and composition varied significantly between clones (Yang et al., 2006). The T-DNA integration into the plant genome which can be linked to the bacterial strain and the number of transferred copies has consequences on the growth and secondary metabolism of the transformed roots. In *Whitania somnifera*, T_L -DNA and T_R -DNA integration frequency was linked to the bacterial strain and had an effect on the transformed material morphology. Typical transformed roots, transformed rooty calluses and transformed calluses were obtained where the whitasteroids level was more related to the material morphology than to the inoculated bacterial strain (Bandyopadhyay et al., 2007). In *Gentiana macrophylla*, transformed roots, T_L -DNA and T_R -DNA integration had an effect on the root specific secoiridoid glucoside gentiopicoside accumulation (Tiwari et al., 2007).

Effect of medium components on growth and metabolite accumulation

The hairy roots growth rate is generally high, but great variations exist from one line to another. Mean doubling time after inoculation ranges from 24 to 90 h (Payne et al., 1991), but sometimes it is much longer. As an example, the doubling time of *Galphimia glauca* hairy roots was 6 days (Nader et al., 2006), and even 15 days in the case of *Cinchona* hairy roots (Geerlings et al., 1999). Optimization of the medium composition may sometimes increase the growth rate of the roots and/or the yield of accumulated metabolites. The use of modified culture media is generally required. These modifications involve changes in sugars, nitrogen, and phosphorous sources. The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *A. belladonna* hairy roots was studied (Bensaddek et al., 2001). An increase of ammonium concentration in the culture medium resulted in lowering the growth rate while an increase of the nitrate concentration had a deleterious effect on the alkaloid biosynthesis and accumulation. The highest biomass and alkaloid yields were obtained with reduced levels of both nitrogen sources. The results obtained by Sivakumar and collaborators with ginseng hairy roots suggest that mineral elements are an important regulatory factor of growth and biomass (Sivakumar et al., 2005).

In vitro culture of plants cells usually require the presence in the medium of plant growth regulators, mainly auxins and cytokinins. In the case of hairy roots, one characteristic of their phenotype is the fast hormone-independent growth. The result is that in media used for the culture of hairy roots hormones are generally lacking. Even more, it has been demonstrated that in transformed roots of *Datura stramonium*, treatment of the cultures with 2.0 mg L^{-1} α -naphthalene acetic acid (NAA) and 0.2 mg L^{-1} kinetin induced a de-differentiation of the root tissue and a redirection of primary nitrogen metabolism (Ford et al., 1996). In several experiments this de-differentiation was accompanied by a significant decrease or even a

cessation of alkaloid production (Robins et al., 1991). However, it was demonstrated more recently that when testing systematically the effect of different types of phytohormones upon root growth and secondary metabolite production, some of them could enhance either growth or metabolites production. In the case of *A. annua* hairy roots (Weathers et al., 2005), the response of cultures to five types of hormones: auxins, cytokinins, ethylene, gibberellins (GA) and abscissic acid (ABA) was evaluated. The highest biomass was obtained when 1-5 mg L⁻¹ ABA was supplied in the medium, while 0.5-1 mg L⁻¹ 2-isopentenyladenine inhibited root growth but stimulated the production of artemisinin more than 2-fold. In other experiments (Yu et al., 2006), *Polygonum multiflorum* hairy root cultures were supplemented with 2,4-D, NAA and 6-BA at various concentrations. The results showed that 0.1 mg L⁻¹ 2-4 D had a deleterious effect on the root cultures; in contrast, NAA and 6-BA in certain conditions could stimulate the growth (0.3-0.4 mg L⁻¹ BA; or 0.4 mg L⁻¹ NAA) and the production of anthraquinones (0.4 mg L⁻¹ BA). With the combined treatment of *P. ginseng* hairy roots with both 25 µM indole-3-butyric acid (IBA) and 100 µM MeJA the productivity of ginsenoside went to 10 mg L⁻¹ d⁻¹, instead of 7.3 mg L⁻¹ d⁻¹ with MeJA alone (Kim et al., 2007).

Elicitation

The use of biotic or abiotic stress on tissue cultures has been shown to have an effect on the secondary metabolite accumulation. The elicitation procedure consists in treating the cultures with a physical or a chemical agent that will cause phytoalexin production leading to defence mechanisms in the plant cells. The eliciting agents are classified in two large categories: abiotic elicitors (physical, mineral and chemical factors), and biotic elicitors which are factors of plant or pathogen origin (Yoshikawa, 1978). As the secondary metabolites are generally produced in nature as a defence mechanism against pathogenic and insect attack, elicitation is often used to enhance their *in vitro* accumulation levels. Elicitation is mainly used when the hairy root cultures have reached its stationary phase, usually around 2-3 weeks after inoculation. There are many recent examples combining hairy root culture and elicitation treatments (Table I), some of which involving the production of pharmacologically-active terpene-derived compounds.

Abiotic elicitors such as NiSO₄ (20 µM), selenium (0.5 mM), and NaCl (0.1%) supplemented in transformed root cultures of *P. ginseng*, increased the saponin content 1.15-1.33 times compared to controls (Jeong et al., 2006). Sodium acetate (10.2 mM), added for 24 h to the culture medium of *Arachis hypogaea* (peanut) hairy roots, lead to a 60-fold induction and secretion of resveratrol and *trans*-resveratrol into the culture medium (Medina-Bolivar et al., 2007). Sorbitol added as an osmoticum had a dramatic effect on tanshinone yield in *Salvia miltiorrhiza* Bunge hairy roots: that yield was increased 4.5-fold as compared to the control (Shi et al., 2007). The effect of biotic elicitors used at higher concentrations (5-400 mg L⁻¹) seems to be clearly efficient. In transformed roots of *P. ginseng*, plant-derived oligosaccharides from *Paris polyphylla* var. *yunnanensis* increased the saponin content by more than 3 times (Zhou et al., 2007). Fungus-derived oligosacchrides (from the fungal endophyte *Colletotrichum gloeosporoides*), yeast elicitor (polysaccharide fraction of the yeast extract), and chitosan increased artemisinin (anti-malarial sesquiterpene endoperoxide) production in *A. annua* 1.5, 3 and 6-fold, respectively (Wang et al., 2006; Putalun et al., 2007). Signal compounds such as salicylic acid and methyl jasmonate (MeJA) can be used as elicitors to enhance the accumulation of secondary metabolites already present in the cultures. In the case of *Azadirachta indica* hairy roots, addition of 100 mM jasmonic acid and salicylic acid showed a 6-9 fold enhancement of azadirachtin, a tetranortriterpenoid with pesticidal activity, as compared to control cultures (Satvide et al., 2007). These elicitors stimulate biosynthetic pathways. In transgenic *Hyoscyamus niger* hairy root cultures over-expressing putrescine N-methyltransferase, MeJA treatment enhanced both polyamine and tropane alkaloid biosynthesis (Zhang et al., 2007). Moreover, added to the medium of *Centella asiatica* at a concentration of 0.1 µM, MeJA triggered *de novo* accumulation of asiaticoside, an anti-inflammatory triterpene saponin which was not initially accumulated in

the hairy roots (Kim et al., 2007). This accumulation followed a linear increase for 2 weeks and could be maintained at its top level (7.12 mg g⁻¹) for one additional week. The same authors also state that the expression of *CabAS*, a putative beta-amyrin synthase, was higher than controls 12 hours after MeJA addition and during the 2 following weeks.

Conclusion and perspectives

At the present time, a constantly increasing number of species have been transformed for the establishment of hairy root cultures: 29 species in 1987, 116 in 1990, and 185 plants from 41 families in 2004 (Kuzovkina and Schneider, 2006). Hairy root cultures offer many advantages among which we can highlight the high and continuous yields of a wide range of metabolites and a high growth potential (Grzegorzyc et al., 2006). The large number of initiated clones offers a screening opportunity (Yu et al., 2006). Large scale culture feasibility and long term stability make this biotechnological approach not only a reliable source of secondary metabolites (Peebles et al., 2007), but also an effective tool to study the biosynthetic pathways of complex plant products (Robins, 1998). However, scaling up hairy roots to industrial levels poses a great challenge at the moment. The efficiency of the scaling up systems still needs optimization before industrial exploitation becomes valuable.

Considering their high efficiency at extremely low concentrations, the use of MeJA and other signalling compounds for transformed root cultures elicitation is opening new ways for a possibly profitable *in vitro* secondary metabolite production. Genetic engineering (Li et al., 2006), nutritional modelling (Cloutier et al., 2007), and cross-species co-culture systems involving hairy roots, are opening exciting future prospects in the field of enhanced production and bioconversion (Lin et al., 2003).

Acknowledgments : the authors would like to thank ECOS- Nord and ANUIES for supporting the cooperation between the french and mexican laboratories on the biotechnological approach of promotion of mexican medicinal plants.

References

- Alfermann AW, Petersen M (1995) Natural product formation by plant cell biotechnology – Results and perspectives. *Plant Cell Tiss Org* **43**:199–205.
- Balunas MJ, Kinghorn AD (2005) Drug discovery from medicinal plants. *Life Sci* **78** :431-441.
- Bandyopadhyay M, Jha S, Tepfer D (2007) Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *Withania somnifera* *Plant Cell Rep* **26**:599–609
- Bensaddek L, Gillet F, Nava-Saucedo JE, Fliniaux MA (2001) The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. *J Biotechnol* **85**:35-40.
- Berlin J (1986) Secondary products from plant cell cultures. In: Rehm HJ & Reed G (eds), *Biotechnology a comprehensive treatise*. Vol.4 pp. 630–658. Verlag Chemie, Verlagsgesellschaft, Weinheim.
- Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux MA, Jacquin-Dubreuil A (2000a) Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes* 15834 and *Agrobacterium tumefaciens* containing *rol* A, B, C genes only. *J Biotech* **81**:151-158
- Bonhomme V, Laurain-Mattar D, Fliniaux MA (2000b) Effects of the *rol* C gene on hairy root : induction development and tropane alkaloid production by *Atropa belladonna*. *J Nat Prod* **62**:1249-1252.
- Butler MS (2005) Natural products to drugs: natural product derived compounds in clinical trials. *Nat Prod Rep* **22**:162-195.

- Cragg GM, Newman DJ, Snader, KM (1997) Natural products in drug discovery and development. *J Nat Prod* **60**:52–60.
- Cragg GM, Newman DJ (2005) Plants as sources of anticancer agents. *J Ethnopharmacol* **100**:72-77.
- Cloutier M, Bouchard-Marchand E, Perrier M, Jolicoeur M (2007) Predictive nutritional model for plant cells and hairy roots. *Biotechnol Bioeng* **99**:189-200.
- Estruch JJ, Chriqui D, Grossmann K, Schell J, Spina A (1991) The plant oncogene *roC* is responsible for the release of cytokinins from glucoside conjugates. *EMBO J* **10**:2889-2895.
- Flores HE, Filner P (1985) Metabolic relationships of putrescine, GABA and alkaloids in cell and root cultures of Solanaceae. In: Neumann KH, Barz W, Reinhard E (eds) Primary and secondary metabolism of plant cell cultures. Springer, Berlin, pp 174–185.
- Ford YY, Ratcliffe RG, Robins RJ (1996) Phytohormone-induced GABA production in transformed root cultures of *Datura stramonium*: an *in vivo* ¹⁵N NMR study. *J Exp Bot* **47**:811-818.
- Gartland JS (1995), *Agrobacterium* virulence. In Gartland KMA, Davey MR, eds, Methods in Molecular biology **44** *Agrobacterium* protocols Humana Press, Totowa New Jersey, pp 15-28.
- Geerlings A, Hallard D, Martinez Caballero A, Lopes Cardoso I, van der Heijden R, Verpoorte R (1999) Alkaloid production by a *Cinchona officinalis* 'ledgeriana' hairy root culture containing constitutive expression constructs of tryptophan decarboxylase and strictosidine synthase cDNA from *Catharanthus roseus*. *Plant Cell Rep* **19**:191-196.
- Gelvin, SB(2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu Rev Plant Phys Plant Mol Biol* **51**:223-256.
- Georgiev MI, Pavlov AI, Bley T. (2007) Hairy root type plant *in vitro* systems as sources of bioactive substances. *Appl Microbiol Biot* **74**:1175-1185.
- Giri A, Narasu ML (2000) Transgenic hairy roots: recent trends and applications. *Biotechnol Adv* **18**:1-22.
- Grzegorzczuk I, Króllicka A, Wysokińska H (2006) Establishment of *Salvia officinalis* L. hairy root cultures for the production of rosmarinic acid. *Z Naturforsch [C]* **61**:351-316.
- Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006 a) Hairy root research : recent scenario and exciting prospects. *Curr Opin Plant Biol* **9**:341-346.
- Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006 b) Harnessing the potential of hairy roots : dawn of a new era. *Trends Biotechnol* **24**:403-409.
- Hamill JD, Parr AJ, Rhodes MJC, Robins RJ, Walton NJ (1987) New routes to plant secondary products. *BioTechnol* **5**:800–804.
- Han XL, Bu HY, Hao JG, Zhao YW, Jia JF (2006) Hairy root induction and plant regeneration of crownvetch (*Coronilla varia* L.) transformed by *Agrobacterium rhizogenes*. *Sheng Wu Gong Cheng Xue Bao* **22**:107-113.
- Hollman A (1996) Drugs and atrial fibrillation. Digoxin comes from *Digitalis lanata*. *Brit Med J* **312**:912
- Hong SB, Peebles CA, Shanks JV, San KY, Gibson SI (2006) Terpenoid indole alkaloid production by *Catharanthus roseus* hairy roots induced by *Agrobacterium tumefaciens* harboring rol ABC genes. *Biotechnol Bioeng* **93**:386-390.
- Jeong GT, Park DH. (2006) Enhanced secondary metabolite biosynthesis by elicitation in transformed plant root system: effect of abiotic elicitors. *Appl Biochem Biotechnol* **129-132**:436-446.
- Kim OT, Bang KH, Shin YS, Lee MJ, Jung SJ, Hyun DY, Kim YC, Seong NS, Cha SW, Hwang B. (2007) Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. *Plant Cell Rep* **26**:1941-1949.
- Kim YS, Yeung EC, Hahn EJ, Paek KY (2007) Combined effects of phytohormone, indole-3-butyric acid and methyl jasmonate on root growth and ginsenoside production in adventitious root cultures of *Panax ginseng* C.A. meyer. *Biotechnol Lett* **29**:1789-1792.
- Komaraiah P, Reddy GV, Reddy PS, Raghavendra AS, Ramakrishna SV, Reddanna P (2003) Enhanced production of antimicrobial sesquiterpenes and lipoxxygenase

- metabolites in elicitor-treated hairy root cultures of *Solanum tuberosum*. *Biotechnol Lett* **25**: 593–597.
- Kumar V, Sharma A, Prasad BCN, Gururaj HB, Ravishankar GA (2006) *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. *Electron J Biotechnol* **9**: 349–357.
- Kuzovkina IN, Schneider B (2006) Genetically transformed root cultures-generation, properties and application in plant sciences. *Prog Bot* **67**:275-314.
- Lam KS (2007) New aspects of natural products in drug discovery. *Trends Microbiol* **15**: 279–289.
- 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.
- Li FX, Jin ZP, Zhao DX, Cheng LQ, Fu CX, Ma F (2006) Overexpression of the *Saussurea medusa* chalcone isomerase gene in *S. involucreta* hairy root cultures enhances their biosynthesis of apigenin. *Phytochemistry* **67**:553-560.
- Lin HW, Kwok KH, Doran PM (2003) Production of podophyllotoxin using cross-species coculture of *Linum flavum* hairy roots and *Podophyllum hexandrum* cell suspensions. *Biotechnol Prog* **19**:1417-1426.
- Lorence A, Medina-Bolivar F and Nessler CL (2004) Camptothecin and 10-hydroxycamptothecin from *Camptotheca acuminata* hairy roots *Plant Cell Rep* **22**: 437-441.
- Matsuki R, Onodera H, Yamauchi T, Uchimiya H (1989) Tissue-specific expression of the *roIC* promoter of the Ri plasmid in transgenic rice plants. *Mol Gen Genet* **22**:12–16.
- Medina-Bolivar F, Condori J, Rimando AM, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan MC (2007) Production and secretion of resveratrol in hairy root cultures of peanut. *Phytochemistry* **68**:1992-2003
- Nader BL, Taketa AT, Pereda-Miranda R, Villarreal ML (2006) Production of triterpenoids in liquid-cultivated hairy roots of *Galphimia glauca*. *Planta Med* **72**:842-844.
- Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. *J Nat Prod* **66**:1022-1037.
- Nilsson O, Olsson O (1997) Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. *Physiol Plantarum* **100**:463–473.
- Oksman-Caldentey, Arroo R (2000) Regulation of tropane alkaloid metabolism in plants and plant cell cultures. In: Verpoorte R & Alfermann AW (eds) *Metabolic engineering of plant secondary metabolism* (pp. 253–281). Kluwer Academic Press, Dordrecht.
- 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
- Payne J, Bringi V, Prince C, Schuler ML (1991) *Plant cell and tissue culture in liquid systems*. Hanser, Munich.
- Peebles CA, Gibson SI, Shanks JV, San KY (2007) Long-Term Maintenance of a Transgenic *Catharanthus roseus* Hairy Root Line. *Biotechnol Prog*, in press.
- Petersen SG, Stummann BM, Olesen P, Henningsen KW (1989) Structure and function of root-inducing (Ri) plasmids and their relation to tumor-inducing (Ti) plasmids. *Physiol Plantarum* **77**:427–435.
- Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempé J (1983) Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol Gen Genet* **190**:204–214.
- Piatczak E, Krolicka A, Wysokinska H (2006) Genetic transformation of *Centaurea erythraea* Rafn by *Agrobacterium rhizogenes* and the production of secoiridoids. *Plant Cell Rep* **25**:1308-1315.
- Putalun W, Luealon W, De-Eknamkul W, Tanaka H, Shoyama Y (2007) Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. *Biotechnol Lett* **29**:1143–1146.

- Raves ML, Harel M, Pang YP, Silman I, Kozikowski AP, Sussman JL (1997) 3D structure of acetylcholinesterase complexed with the nootropic alkaloid, (-)-huperzine A. *Nat Struct Biol* **4**: 57–63.
- Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, Fridlender B (2002) Plants and human health in the twenty-first century. *Trends Biotechnol* **20**: 522-531.
- Robins RJ (1998) The application of root cultures to problems of biological chemistry. *Nat Prod Rep* **15**: 549-570.
- Robins RJ, Bent EG, Rhodes MJC (1991) Studies on the biosynthesis of tropane alkaloids by *Datura stramonium* L. transformed root cultures. 3. The relationship between morphological integrity and alkaloid biosynthesis. *Planta* **185**: 385-390.
- Samuelsson, G., 2004. *Drugs of Natural Origin: a Textbook of Pharmacognosy*, 5th edition Swedish Pharmaceutical Press, Stockholm.
- Satdive RK, Fulzele DP, Eapen S. (2007) Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* A. Juss by elicitation and media optimization. *J Biotechnol* **128**:281-289.
- Schmülling T, Schell J, Spena A (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J* **7**:2621–2629.
- Schmidt JF, Moore MD, Pelcher LE, Covello PS (2007) High efficiency *Agrobacterium rhizogenes*-mediated transformation of *Saponaria vaccaria* L. (Caryophyllaceae) using fluorescence selection. *Plant Cell Rep* **26**: 1547-1554.
- Scott LJ, Goa KL (2000) Galantamine: a review of its use in Alzheimer's disease. *Drugs* **60**: 1095–1122.
- Sevon N, Oksman-Caldentey K-M (2002) *Agrobacterium rhizogenes* mediated transformation: root cultures as a source of alkaloids. *Planta Med* **68**:859–868
- Shi M, Kwok KW, Wu JY. (2007) Enhancement of tanshinone production in *Salvia miltiorrhiza* Bunge (red or Chinese sage) hairy-root culture by hyperosmotic stress and yeast elicitor. *Biotechnol Appl Biochem* **46**:191-196.
- Shi HP, Qi Y, Zhang Y, Liang S (2006) Induction of cucumber hairy roots and effect of cytokinin 6-BA on its growth and morphology *Sheng Wu Gong Cheng Xue Bao* **22**:514-520.
- Sivakumar G, Yu KW, Hahn EJ, Paek KY (2005) Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors. *Current Sci* **89**: 641-649.
- Stachel SE, Messens E, van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. *Nature* **318**: 624-629.
- Srivastava S, Srivastava AK(2007) Hairy root culture for mass-production of high-value secondary metabolites. *Crit Rev Biotechnol* **27**:29-43.
- Tao J, Li L (2006) Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes*. *South Afr J Bot* **72**:211-216
- Tiwari RK, Trivedi M, Guang ZC, Guo GQ, Zheng GC (2007) Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. *Plant Cell Rep* **26**:199-210.
- Tomilov A, Tomilova N, Yoder JI (2007) *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* transformed roots of the parasitic plant *Triphysaria versicolor* retain parasitic competence. *Planta* **225**:1059–1071.
- Trick HN, Finer JJ (1997) SAAT: Sonication-Assisted *Agrobacterium*-mediated transformation. *Transgenic Res* **6**: 329–336.
- van Agtmael, M.A., Eggelte, T.A., van Boxtel, C.J., 1999. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trends Pharmacol Sci* **20**:199–205.
- Vanhala L, Hiltunen R, Oksman-Caldentey KM (1995) Virulence of different *Agrobacterium* strains on hairy root formation of *Hyoscyamus muticus*. *Plant Cell Rep* **14**: 236–240.
- Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant

- secondary metabolites. *Phytochem Rev* **1**: 13-25.
- Wang JW, Kong FX, Tan RX (2002) Improved artemisinin accumulation in hairy root cultures of *Artemisia annua* by (22S, 23S)-homobrassinolide. *Biotechnol Lett* **24**: 1573–1577.
- Wang JW, Zheng LP, Tan RX. (2006) The preparation of an elicitor from a fungal endophyte to enhance artemisinin production in hairy root cultures of *Artemisia annua* L. *Sheng Wu Gong Cheng Xue Bao* **22**: 829-834.
- Webb KJ, Jones S, Robbins MP, Minchin FR (1990) Characterization of transgenic root cultures of *Trifolium repens*, *Trifolium pratense* and *Lotus corniculatus* and transgenic plants of *Lotus corniculatus*. *Plant Sci* **70**: 243-254.
- Wheathers PJ, 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
- Yang SH, Liu XF, Guo DA, Zhen JH (2006) Induction of hairy roots and anthraquinone production in *Rheum palmatum* *Zhongguo Zhong Yao Za Zhi* **18**:1496-1499.
- Yoshikawa M (1978) Diverse modes of action of biotic and abiotic phytoalexin elicitors. *Nature* **275**: 546–547.
- Yu RM, Ma N, Yan CY, Zhao Y (2006) Effects of exogenous phytohormones on hairy root growth of *Polygonum multiflorum* and biosynthesis of anthraquinones in its hairy root cultures. *Chin J Biotech* **22**: 619-623.
- Yu SH, Zha JP, Zhan WH, Zhang DQ. (2006) Contents comparison of resveratrol and polydatin in the wild *Polygonum cuspidatum* plant and its tissue cultures. *Zhongguo Zhong Yao Za Zhi* **31**:637-641.
- Yue-Zhong Shu, 1998. Recent natural products based drug development: a pharmaceutical industry perspective. *J Nat Prod* **61**:1053–1071.
- Zhang L, Yang B, Lu B, Kai G, Wang Z, Xia Y, Ding R, Zhang H, Sun X, Chen W, Tang K. (2007) Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing putrescine N-methyltransferase is methyl jasmonate-dependent. *Planta* **225**:887-896.
- Zhou L, Cao X, Zhang R, Peng Y, Zhao S, Wu J. (2007) Stimulation of saponin production in *Panax ginseng* hairy roots by two oligosaccharides from *Paris polyphylla* var. *yunnanensis*. *Biotechnol Lett* **29**:631-634.
- Zupan J, Zambryski P (1997) The *Agrobacterium* DNA transfer complex. *Crit Rev Plant Sci* **16**: 279-295.

Type of elicitation	Species	Produced metabolites and medicinal properties	Elicitor	Fold increase of the metabolite content ^a	Ref.
Abiotic	<i>Panax ginseng</i>	Total saponin content tonic, stimulant, adaptogenic	NiSO ₄ 20 µM	1.2 - 1.23	Jeong et al., 2006
			Selenium 0.5 mM	1.31 - 1.33	
			NaCl 1%	1.13 - 1.15	
Abiotic	<i>Salvia miltiorrhiza</i>	Tanshinone antioxidant anti-inflammatory	Sorbitol 50 g L ⁻¹	4.5	Shi et al., 2007
	<i>Arachis hypogaea</i>	Resveratrol and <i>trans</i> -resveratrol antioxidant, atherosclerosis prevention	Sodium acetate 10.2 mM	60	Medina-Bolivar et al., 2007
	<i>Panax ginseng</i>	Total saponin content	Oligosaccharides from <i>Paris polyphylla</i> 30 mg L ⁻¹ , plant derived	3	Zhou et al., 2007
Biotic	<i>Artemisia annua</i>	Artemisinin antimalarial	Oligosaccharides from <i>Colletotricum gloeosporoides</i> 0.4 mg total sugar mL ⁻¹ , fungus derived.	1.51	Wang et al., 2006 Putalun et al., 2007
			Polysaccharide fraction of the yeast extract 2 mg L ⁻¹	3	
			Chitosan 150 mg L ⁻¹	6	
	<i>Azadirachta indica</i>	Azadirachtin pesticidal	Salicylic acid 100 mM	6	Satvide et al., 2007
			Jasmonic acid 100 mM	9	
	<i>Hyoscyamus niger</i> , PMT overexpression	Polyamines and tropane alkaloids mydriatic, parasympatholytic, antiparkinsonian	MeJA 50 µM	2	Zhang et al., 2007
<i>Centella asiatica</i>	Asiaticoside anti-inflammatory	MeJA 0.1 µM	<i>de novo</i> accumulation	Kim et al., 2007	

Table I : Elicitation of hairy root cultures accumulating pharmacologically-active compounds.
^a ratio as compared to the content of not elicited hairy roots.