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

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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*, vincristrine 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) chimiotactism 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), *Centaurium 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 decarboxylaxe (*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 µM) 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 µM.

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 gentiopicroside 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-naphtalene 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 $NiSO_4$ (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. yunanensis 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 nig*er 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 (Grzegorczyc 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.

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Type of elicitation	Species	Produced metabolites and medicinal properties	Elicitor	Fold increase of the metabolite content [a]	Ref.
Abiotic	*Panax ginseng*	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	
	Salvia miltiorrhiza	Tanshinone antioxidant anti-inflammatory	Sorbitol 50 g L^{-1}	4.5	Shi et al., 2007
	Arachis hypogaea	Resveratrol and *trans*-resveratrol antioxidant, atherosclerosis prevention	Sodium acetate 10.2 mM	60	Medina-Bolivar et al., 2007
Biotic	*Panax ginseng*	Total saponin content	Oligosaccharides from *Paris polyphylla* 30 mg L^{-1}, plant derived	3	Zhou et al., 2007
	Artemisia annua	Artemisinin antimalarial	Oligosaccharides from *Colletotricum gloeosporoides* 0.4 mg total sugar mL^{-1}, fungus derived.	1.51	Wang et al., 2006
			Polysaccharide fraction of the yeast extract 2 mg L^{-1}	3	Putalun et al., 2007
			Chitosan 150 mg L^{-1}	6	
	Azadirachta indica	Azadirachtin pesticidic	Salicylic acid 100 mM	6	Satvide et al., 2007
			Jasmonic acid 100 mM	9	
	Hyoscyamus niger, PMT overexpression	Polyamines and tropane alkaloids mydriatic, parasympatholytic, antiparkinsonian	MeJA 50 µM	2	Zhang et al., 2007
	Centella asiatica	Asiaticoside anti-inflammatory	MeJA 0.1 µM	*de novo* 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.