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# *Agrobacterium rhizogenes*-Mediated Transformation: Root Cultures as a Source of Alkaloids



**Abstract**

Hairy roots, transformed with *Agrobacterium rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions. A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes*. Transformed root cultures have also been found to be a potential source of high-value pharmaceuticals. In this article the most important alkaloids produced by hairy roots are summarised. Several different methods have been used to increase the alkaloid accumulation in hairy root cultures. The selection of high productive root lines based on somaclonal variation offers an interesting option to enhance the productivity. Elicitors and modification of culture conditions have been shown to increase the growth and the alkaloid production in some cases. Genetic engineering is a modern tool to regulate the secondary metabolism also in hairy roots. However, our knowledge on biosynthesis of many alkaloids is still poor. Only a limited number of enzymes and their respective genes which regulate the biosynthetic pathways are fully characterised.

**Key words**
*Agrobacterium rhizogenes* · alkaloids · genetic engineering · hairy roots · secondary metabolites



## Introduction

One of the most studied group of secondary metabolites is alkaloids. Today more than 16.000 alkaloids have been identified from plants [1]. Alkaloids also represent an important pharmaceutical and economical value, and many of them are currently isolated from plant material since no alternative production method exists which is economically feasible. For the past few decades considerable interest has been shown in the production of the secondary products by plant cell cultures [2]. The success, however, has been moderate mainly because the biosynthesis pathways of the secondary metabolites, including the enzymes and the regulatory mechanism governing expression and function of the pathways, are poorly understood [3], [4]. Secondary product formation is often low and unstable in undifferentiated callus and suspension cultures; the metabolism of secondary products seems to correlate with the degree of organisation of cell structures. Therefore, the roots are capable of accumulating a large range of secondary metabolites reflecting biosynthetic capacity [5]. As an example several alkaloids that are scarcely synthesised in undifferentiated cells are produced at relatively high levels in cultured roots. This suggests that alkaloid production is associated with the root organogenesis [6]. The major problem associated with the *in vitro* culture of conventional roots is usually the slow growth rate. In contrast, *Agrobacterium*-mediated hairy roots are fast-growing and genetically stable which can also be successfully cultured in large-scale bioreactors (e.g., [7]). Transformed roots are able to produce secondary metabolites at levels that are often comparable to, or greater than that of the intact plants [2], [8], [9].

In this review we describe *Agrobacterium rhizogenes*-mediated transformation of plants to obtain hairy root cultures, recent achievements and improvements on alkaloid production in

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**Received** February 12, 2002 · **Accepted** May 25, 2002

**Bibliography**
Planta Med 2002; 68: 859–868 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943

transformed roots, and finally the possibilities for metabolic engineering using hairy root system.

## Hairy Root Disease

Hairy root and the crown gall tumor are two plant diseases caused by two Gram-negative soil bacteria, *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively. Depending on the strains of *Agrobacterium* involved, one or both of the two pieces (TL and TR) of the Ti-(tumor inducing) or Ri- (root inducing) plasmid are transferred from the bacteria into the nuclear genome of the host plant. The transferred T-DNA derived from the Ti-plasmid causes the plant cells to proliferate, to form crown gall tumors, and in the case of the Ri-plasmid, the extensive formation of adventitious roots at or near the site of infection. The transformed plant tissues are also directed by T-DNA genes to produce unusual metabolites called opines, that serve as specific nutrients for the bacteria [10], [11].

The molecular mechanism of T-DNA transfer to the plant is most likely the same for both *Agrobacterium* species, but the physiological basis of tumorigenesis is totally different. Crown gall tumors result from the overproduction of the phytohormones auxin and cytokinin specified by *A. tumefaciens* T-DNA genes [12]. The physiological basis of the hairy root disease is not totally understood. Alteration of auxin metabolism in transformed cells has been supposed to play an important role in expression of the hairy root phenotype [12], [13]. TL-DNA plays the major role in hairy root induction, and the genes encoding auxin synthesis have a somewhat accessory role [14], [15]. Auxin is necessary for hairy root induction, but it seems likely that auxin does not play a role in T-DNA expression in transformed plant cells [14]. Physiological studies have indeed shown that the transformed cells are more sensitive to extracellularly supplied auxins than the normal roots [16]. Spanó and co-workers [17] have suggested that the genes responsible for increased sensitivity of hairy root cells to auxin are located on the TL-DNA. On the other hand, hairy roots of *Hyoscyamus muticus* L. have been demonstrated to tolerate high auxin levels. The sensitivity is most probably restricted to certain plant species [18].

### Characterization of *Agrobacterium* plasmids

Several classes of both Ri- and Ti-plasmids have been characterised. The plasmids are large (200 to greater than 800 kb) and contain one or two regions of T-DNA and a *vir* (virulence) region, all of which are necessary for tumorgenesis [12], [19]. The classification of plasmids depends to a large extent on the type of opines that the plasmids direct the infected plants to synthesise. The Ri-plasmids are grouped into two main classes according to the opines synthesised by hairy roots. Agropine-type strains (e.g., A4, 15834, LBA9402, 1855) induce roots to synthesise agropine, mannopine and the related acids, and mannopine,-type strains (e.g., 8196, TR7, TR101) induce roots to produce mannopine and the corresponding acids [20]. Other types of opines (e.g., cucumopine, mikimopine) have also been described [21], ]22].

The agropine-type Ri-plasmids are very similar as a group, and a quite distinct group from the mannopine-type plasmids [23]. Perhaps the most studied Ri-plasmids are agropine-type strains, which are considered to be the most virulent and therefore more often used in the establishment of hairy root cultures [24].

The plasmids of *A. tumefaciens* were used as vectors for many years before the interaction between *Agrobacterium rhizogenes* and the plant cell was discovered [10], [19], [25]. The use of *Agrobacterium* as a vector is based on its unique capacity to transfer a piece of its own DNA (T-DNA) into the nuclear genome of plant cells. Any DNA placed between the borders will be transferred to a plant cell. This property has been extremely useful for the introduction of new genes into plants, either for research or for practical applications [26].

### The genes responsible for hairy root formation

The T-DNA of the agropine-type Ri-plasmid consists of two separate T-DNA regions designed the TL-DNA and TR-DNA [23]. Each of the T-DNA fragments spans a 15 – 20 kb region, and they are separated from each other by at least 15 kb of non-integrated plasmid DNA. These two fragments can be transferred independently during the infection process [27]. White and co-workers [23] made a comparison between the T-DNA region of the agropine and mannopine-type Ri-plasmids and the octopine and nopaline-type Ti-plasmids. The agropine-type Ri T-DNA has limited sequence homology to either the nopaline- or octopin-type Ti-DNA sequences, and share homology only in the region corresponding to two loci encoding auxin synthesis and the agropine synthesis loci [23]. No homology has been found between Ri T-DNA and the *tmr* locus of the Ti T-DNA. The genes encoding auxin synthesis (*tms*1 and *tms*2) and agropine synthesis (*ags*) have been localised on the TR-DNA of the agropine type Ri-plasmid [23], [28]. The mannopine type Ri-plasmids contain only one T-DNA that shares considerable DNA sequence homology with TL of the agropine-type plasmids [12].

Mutation analysis of the TL-DNA has led to identification of four genetic loci, designed locus *rol*A, *rol*B, *rol*C, and *rol*D, which affect hairy root induction [15], [23]. The complete nucleotide sequence of the TL-region revealed the presence of 18 open-reading frames (OFRs), 4 of which, ORFs 10, 11, 12 and 15, respectively, correspond to the *rol*A, *rol*B, *rol*C, and *rol*D loci. It was also shown that *rol*A, *rol*B, and *rol*C play the most important role in hairy root induction. In particular, *rol*B seems to be the most crucial in the differentiation process of transformed cells, while *rol*A and *rol*C provide with accessory functions [15].

Although the TR-DNA is not essential for hairy root formation it has been shown that the *aux*1 gene harboured in this segment provides to the transformed cells with an additional source of auxin. Recently, Moyano and co-workers [29] found that *aux* genes play a significant role in the morphology and alkaloid production of transformed roots of *Datura metel* and *Duboisia* hybrid. The studies with *Panax ginseng* c.v. Meyer hairy roots also support this finding (Mallol et al., unpublished results).

### Mechanism of *Agrobacterium*-plant cell interaction

One of the earliest stages in the interaction between *Agrobacterium* and a plant is the attachment of the bacterium to the surface of the plant cell. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone, that activate the

*vir*-region of the bacterial plasmid [30]. It has been shown that the *Agrobacterium* plasmid carries three genetic components that are required for plant cell transformation [13]. The first component, the T-DNA that is integrated into the plant cells, is a mobile DNA element. The second one is the virulence area (*vir*), which contains several *vir* genes. These genes do not enter the plant cell but, together with the chromosomal DNA (two loci), cause the transfer of T-DNA. The third component, the so-called border sequences (25 bp), resides in the *Agrobacterium* chromosome. The mobility of T-DNA is largely determined by these sequences, and they are the only *cis* elements necessary for direct T-DNA processing.

Zupan and Zambryski [31] have described in details the mechanism for the transfer of T-DNA into the plant cell. The early steps of the transfer are relatively well studied but the mechanics of integration are not completely understood. According to Zambryski [32], it is a multistep process involving recombination, replication and repair activities, most likely mediated by host cell enzymes. The overall process of integration is probably very similar to any illegitimate recombination of foreign DNA into eukaryotic genomes [26].

Comprehensive reviews on *Agrobacterium* transformation can be found in Zhu and co-workers [19] and Zupan and co-workers [25].

## Establishment of Hairy Root Cultures

The transformation is induced on aseptic, wounded plants or plant parts by inoculating them with a thick, viable *A. rhizogenes* suspension. After 1 – 4 weeks, when roots emerge at the site of inoculation, they are individually cut off and transferred into a hormone-free growth medium e. g., MS [33] or B5 [34], containing antibiotics to kill the bacteria. The protocol of the establishment of hairy root cultures has been described in detail by [8], [35], [36].

The susceptibility of plant species to *Agrobacterium* strains varies greatly. However, plant species, which were shown to be insusceptible to *A. rhizogenes*, e. g., strain A4, have been successfully transformed with other strains [37], [38]. Significant differences were observed between the transformation ability of different strains of *Agrobacterium* [36], [39]. The age and differentiation status of plant tissue can also affect the chances of successful transformation. The level of tissue differentiation also determines the ability to give rise to transformed roots after *A. rhizogenes* inoculation [40]. Successful infection of some species can be achieved by the addition of acetosyringone [41].

The genetic transformation can be confirmed by assaying the opines. Opine production can, however, be unstable in hairy roots and may disappear after a few passages [42]. For this reason, detection of T-DNA by Southern blot hybridization is often necessary to confirm the genetic transformation [43]. The polymerase chain reaction (PCR) simplifies the detection of transformation [44], [45].

## Characteristics of the Hairy Roots Cultures

Hairy roots are fast growing and laterally highly branched, and are able to grow in hormone-free medium. Moreover, these organs are not susceptible to geotropism anymore. They are genetically stable and produce high contents of secondary metabolites characteristic to the host plant. The secondary metabolite production of hairy roots is stable compared to other types of plant cell culture. The alkaloid production of hairy roots cultures has been reported to remain stable for years [6]. The secondary metabolite production of hairy roots is highly linked to cell differentiation. Alkaloid production decreased clearly when roots were induced to form callus, and reappeared when the roots were allowed to redifferentiate [6], [42]. An interesting characteristic of some hairy roots is their ability to occasionally excrete the secondary metabolites into the growth medium [46]. However, the extent of secondary product release in hairy root cultures varies between species [47], [48], [49].

The average growth rate of hairy roots varies from 0.1 to 2.0 g dry weight/litre/day. This growth rate exceeds that of virtually all-conventional roots and is comparable with that of suspension cultures. However, the greatest advantage of hairy roots compared to conventional roots is their ability to form several new growing points and, consequently, lateral branches [2]. The growth rate of hairy roots may vary greatly between species, but differences are also observed between different root clones of the same species [6], [50], [51], [52]. The pattern of growth and secondary metabolite production of hairy root cultures can also vary. Secondary production of the hairy roots of *Nicotiana rustica* L. was strictly related to the growth, whereas hairy roots of *Beta vulgaris* L. exhibited non-growth-related product accumulation [47]. In the case of the hairy roots of *Scopolia japonica* Jacq. and *H. muticus*, the secondary products only started to accumulate after growth had ceased [36], [46], [53]. Secondary metabolite synthesis dissociated from growth would be desirable for commercial production, as it would allow the use of continuous systems.

## Improvement of the Production in Transformed Root Cultures

Hundreds of plant species have been successfully transformed to hairy roots (see the reviews by Tepfer [54] and Giri and Narasu [9]). For the past ten years hairy roots have also been investigated as a potential source of pharmaceuticals [2], [9]. Table **1** summarises some of the most important alkaloids produced by the hairy root cultures of medicinal plants. The comparison of hairy roots is not always possible, since the product yield is calculated in many different ways (e. g., mg/g f. w. or d. w., mg/flask, % of d. w., mg/l), and all the parameters are not always given. Additionally, high contents of the product could be associated with poor growth, and thus the real productivity (mg/l) remains low.

The secondary metabolites of hairy roots are strictly limited to those that are normally produced in the roots. On the other hand, if the biosynthesis of secondary metabolites normally takes place in the green parts of plants, it is necessary to utilise modified hairy roots, e. g., "green hairy roots" or, alternatively,

Table **1** Alkaloid production of the hairy root cultures of some medicinal plants

| *Plant* | *Alkaloid* | *Content (mg/d d. w.)* | *Reference* |
|---|---|---|---|
| *Aconitum heterophyllum* | aconites | 29.6 | [102] |
| *Atropa belladonna* | atropine | 3.7 | [103] |
| | cuscohygrine | 2.8 | [37] |
| | *l*-hyoscyamine | 9.5 | [37] |
| | scopolamine | 3.0 | [98] |
| | atropine | 7.6 | [104] |
| | scopolamine | 0.3 | [104] |
| | littorine | 0.9 | [105] |
| *Brugmansia candida* | scopolamine | 2.5 mg/g f. w. * | [83] |
| | hyoscyamine | 1.0 mg/g f. w. * | [83] |
| | scopolamine | 0.26* | [106] |
| | hyoscyamine | 0.86* | [106] |
| *Catharanthus roseus* | ajmalicine | 4.0 | [51] |
| | catharanthine | 2.0 | [51] |
| | serpentine | 2.0 | [51] |
| | vindoline | 4.0 | [51] |
| | vinblastine | 0.003 µg/g f. w. | [107] |
| *Catharanthus tricophyllus* | crude alkaloids | 9.2 | [88] |
| *Calystegia sepium* | cuscohygrine | 3.0 | [37] |
| *Cinchona ledgerina* | cinchonine | 1.6 µg/g f. w. | [38] |
| | cinchonidine | 18.0 µg/g f. w. | [38] |
| | quinidine | 15.9 µg/g f. w. | [38] |
| | quinine | 24.3 µg/g f. w. | [38] |
| *Cinchona officinalis* | cinchonine + | | |
| | cinchonidine | 0.4 | [107], [108] |
| | quinidine | 1.0 | [107], [108] |
| | quinine | 0.5 | [107], [108] |
| | strictosidine | 1.9 | [107], [108] |
| *Datura candida* | scopolamine | 5.7 | [109] |
| | *l*-hyoscyamine | 1.1 | [109] |
| *Datura innoxia* | *l*-hyoscyamine | 1.7 | [110] |
| *Datura metel* | scopolamine | 4.1* | [85] |
| | hyoscyamine | 1.0* | [85] |
| *Datura stramonium* | *l*-hyoscyamine | 5.6 | [111] |
| | scopolamine | 5.6 | [112] |
| | hyoscyamine | 6.4 | [113] |
| | scopolamine | 1.9 | [113] |
| *Duboisia hybrid* | *l*-hyoscyamine | 2.1 | [110] |
| | scopolamine | 2.5 | [110] |
| *Duboisia leichhardtii* | scopolamine | 18.0 | [50] |
| *Duboisia myoporoides* | *l*-hyoscyamine | 8.0 | [35] |
| | scopolamine | 2.4 | [35] |
| | scopolamine | 32.0 | [62] |
| *Hyoscyamus albus* | *l*-hyoscyamine | 8.0 | [114] |
| | scopolamine | 4.6 | [110] |
| | hyoscyamine | 15.1 | [76] |
| | scopolamine | 5.4 | [76] |
| *Hyoscyamus niger* | *l*-hyoscyamine | 12.5 | [110] |
| *Hyoscyamus muticus* | *l*-hyoscyamine | 12.2 | [6] |
| | atropine | 1.8 | [76] |
| | scopolamine | 1.0 | [99] |
| *Nicotiana tabacum* | nicotine | 1.1 mg/g f. w. | [48] |
| | nicotine | 0.1 mg/g f. w. | [115] |
| *Nicotiana rustica* | nicotine | 0.3 mg/g f. w. | [63] |
| | anatabine | 0.4 mg/g f. w. | [63] |
| | nicotine | 0.9 mg/g f. w. * | [116] |
| *Peganum harmala* | β-carbolines | 17.0 | [117] |
| *Scopolia carniolica* | *l*-hyoscyamine | 2.0 | [111] |
| *Scopolia japonica* | *l*-hyoscyamine | 13.0 | [118] |
| | scopolamine | 5.0 | [118] |
| *Scopolia tangutica* | *l*-hyoscyamine | 0.5 | [110] |
| | scopolamine | 0.2 | [110] |
| *Solanum tuberosum* | steroidal alkaloids such as solanine | 0.1 mg/g f. w. | [56] |
| *Weigelia* "Styrica" | ajmalicine | 1.4 µg/g | [119] |
| | serpentine | 0.2 µg/g | [119] |

* calculated from the figure

transformed shoot teratomas [42], [55]. Conversely, the shoot teratomas of *Nicotiana tabacum* L., *A. belladonna* L. and *Solanum tuberosum* L. failed to produce alkaloids, indicating that the biosynthesis site of these alkaloids is in the roots [56].

**Selection of high-producing cell lines**

Somaclonal variation has been used widely as a breeding tool in the search for agriculturally interesting traits. Cultured plant cells are heterogeneous and it is therefore possible to select the cells with respect to a particular desired property [57]. The selection of highly productive cell lines has for long been a well-known strategy for the production of secondary metabolites by cell cultures. Considerable somaclonal variation was found in cell cultures derived from protoplasts of *H. muticus* [58], [59]. Thus high alkaloid-producing plants can be also obtained by selection [60]. However, there is no complete agreement on the reasons for the diversity in alkaloid production among clones. Somaclonal variation is caused by genetic changes, which may alter the gene expression of the cells and the synthesis of secondary metabolites. Genetic changes certainly cause the occasional variability in secondary metabolites, but the expression of many secondary pathways is easily altered by external factors and, furthermore, the responses of the cells to external factors depend on their physiological stage. However, a different level of a particular metabolite is the result of differential and reversible gene expression [61].

Mano and co-workers [50] derived forty-five hairy root clones of *D. leichhardii* F.v.M. from individual root meristems and found that there was considerable variation in growth rate, alkaloid content and productivity between the clones. Generally hairy roots are considered to be stable and not easily manipulated. However, hairy roots also possess a certain amount of heterogeneity even though derived from a single root tip, because repeated selection has shown to be applicable to hairy root cultures in order to obtain high scopolamine-producing hairy root lines [62]. Nicotinic acid can be used as a selective agent in order to isolate high nicotine-producing root lines of *N. rustica* hairy root cultures. However, the selected root clones also had a higher ability to detoxify nicotinic acid to nicotine and anatabine [63]. Amino acid analogues have also been used for establishing hairy root lines with a high yield of *l*-hyoscyamine [64].

Protoplasts also offer a possibility to isolate high-producing variants at the single cell level. There are some reports of protoplasts having been isolated from root material [65]. Selected plants with high contents of the desired products should be used as the starting material for protoplast isolation [58]. Statistically high-producing plants give rise to high producing cell lines. The hairy root clones of *N. rustica* regenerated from protoplasts showed variation in morphology, alkaloid formation and T-DNA structure. Some clones also showed increased alkaloid production [66]. Clear differences were also observed in the growth rate, morphology and in the *l*-hyoscyamine content between the protoplast-derived hairy root clones of *H. muticus*. Most of the protoplast-derived hairy root clones showed increased alkaloid synthesis characteristics compared to the parent line. The *l*-hyoscyamine content ranged from 0.04% to 1.5%. The mean content of the clones (0.49%) was, however, almost the same as in that the parent clone (0.57%) that was used as the starting material for the protoplast isolation [65]. The most comprehensive study on somaclonal variation in transformed roots and protoplast derived hairy root clones has been performed by Sevón and co-workers [6]. They could show that the clones were stable over long-term cultivation and the large variation between the clones remained several years unchanged.

**Optimizing the growth conditions and the medium**

Several physical and chemical factors have been found that could influence the growth and productivity of hairy root cultures. However, hairy roots are not so easily modified by changing the culture conditions as cell suspension cultures [67]. Several studies have been made on the effect of medium composition on growth and the production of secondary metabolites. Most of the investigations have been carried out with hairy roots of *Catharanthus roseus* L. [68] and *Solanaceous* species [52], [69].

Factors such as the carbon source and its concentration, ionic concentration of the medium [70], pH of the medium [71], light [70], phytohormones [18], [72], [73], temperature [74] and inoculum [8], [50], [75], are known to influence the growth and alkaloid production of hairy roots. *A. rhizogenes* strain could also have effect on biomass and alkaloid productivity of hairy roots. The atropine yield of root lines of *Hyoscyamus albus* L. induced by *A. rhizogenes* strain A4 were significantly higher than the root lines of *H. albus* induced by *A. rhizogenes* strain LBA9402. Such relationship between the bacterial strain and alkaloid productivity could not be found in case of root lines of *H. muticus* [76].

Gamborg's B5 medium is the most widely used medium for the hairy roots of many species [69]. Supplementation of heavy metal ions, such as $Cu^{2+}$, has been shown to stimulate alkaloid production [46], [70]. Concentrations of inorganic phosphate above or below that present in Gamborg B5 medium (1.0 mM) has reduced the cell yield of hairy root cultures of *Datura stramonium* L., but low levels of phosphate stimulated *l*-hyoscyamine production. Nitrate also reduced cell yield and *l*-hyoscyamine production at concentrations above that present in Gamborg B5 medium (30 mM) [77].

Toivonen and co-workers [68] studied the effect of varying concentrations of sucrose, phosphate, nitrate and ammonium on growth and indole alkaloid production in hairy root cultures of *C. roseus*. They found that low nutrient levels enhanced alkaloid production, but biomass yields were maximal in media containing high concentrations of sucrose and ammonia. Similar results have been obtained by Payne and co-workers [77], who reported that the optimum concentrations of phosphate and nitrate for product formation were lower than that for growth.

Hairy root cultures of *H. muticus* also produced the highest *l*-hyoscyamine content at a sucrose concentration of 30 g/l, but higher than this stimulated the growth of the hairy roots. The root clones of *H. muticus* could not utilise ammonium as the sole nitrogen source. Maximum growth and *l*-hyoscyamine production was achieved when the content of ammonium was not more than 2 mM [52]. Ammonium had a strong influence on the growth of hairy roots of *A. belladonna* while nitrate had clear effect on the alkaloid production and the scopolamine and hyoscyamine ration [78]. Modifying the culture conditions can increase the growth rates and biomass yields of the hairy roots of

*D. stramonium*. However, the specific extracellular productivity (mg alkaloid /g biomass) cannot be significantly increased by varying either the temperature or the relative nutrient levels of sucrose and minerals in the medium [70], [74].

The hairy roots of different species behave differently in the same culture conditions. Hilton and Wilson [69] investigated the growth and uptake of sucrose and minerals ions by six tropane alkaloid-producing transformed root cultures and found that their requirements for certain mineral ions varied when grown in batch cultures on Gamborg's B5 medium. Individual hairy root clones can also have different optimum concentrations of sucrose or mineral ions [52]. The different requirements make optimisation work difficult, because the culture conditions have to be optimised separately for each species and for individual clones.

**Effect of elicitors**

Elicitation is one of the methods that have been used to enhance secondary metabolites of cell cultures [79]. Not many publications have appeared on the elicitation of root or hairy root cultures, most of the results being from experiments with cell suspension cultures [67[, [80]. Table **2** lists the reports where elicitors have been applied to hairy root cultures of medicinal plants. There are only a limited number of alkaloids whose production can be induced by elicitors. Those compounds, which defend the plants against micro-organisms, namely, phytoalexins, are often easily formed in response to the elicitors, but the accumulation of the alkaloids of interest has not usually been induced.

Although the use of elicitors does not directly increase the alkaloid content of hairy roots, cell permeability increases and this often has a positive effect on the formation of secondary metabolites [81]. The fungal elicitors and agents that increase the excretion of desired compounds have on occasions been combined successfully in the treatment of hairy roots of *C. roseus* [82].

Enhancement of cell permeability may increase the formation of secondary products, because feedback inhibition and intracellular degradation of the products decrease. The economical benefit of the production process also depends on the capacity of the producing cells to secrete the desired metabolite into the surrounding medium. Permeability of plant membranes for the release of secondary metabolites has often been connected with the loss of viability of the plant cells treated with permeabilizing agents and methods [81]. Some attempts have been made to increase the permeability of the hairy roots. Biotic and abiotic elicitors including solvents and detergents have been reported to release the products from hairy roots into the medium without any loss of viability and production capacity of the hairy roots [82], [83], [84]. Cusidó and co-workers [85] reported that tween 20 treatment encouraged both growth and alkaloid productivity of hairy roots of *Datura metel* L. Additionally tween 20 treatment clearly increased the extracellular content of scopolamine.

Chitosan has been used as an effective elicitor, but it also enhances the permeability of the cells [46], [86], [87]. Permeabilization studies with chitosan have mainly been performed with

Table **2** Some elicitation studies carried out with the hairy root cultures of the medicinal plants

| *Hairy roots* | *Elicitor* | *Effect* | *Reference* |
|---|---|---|---|
| *Brugmansia candida* | Hemisellulase<br>Theophylline<br>$CaCl_2$ | Stimulation of hyoscyamine and scopolamine | [83] |
| *Brugmansia candida* | Salicylic acid<br>Yeast extract<br>$CaCl_2$<br>$AgNO_3$<br>$CdCl_2$ | Stimulation of hyoscyamine and scopolamine | [84] |
| *Catharanthus roseus* | Methyl jasmonate | Stimulation of catharanthine and ajmalicine | [120] |
| *Catharanthus roseus* | *Penicillium* sp homogenate | Stimulation of catharanthine and ajmalicine | [121] |
| *Datura stramonium* | Wide range of abiotic elicitors (metal ions) | Accumulation of sesquiterpene phytoalexins (lubimin, 3-hydroxylubimin, rishitine) | [122] |
| *Hyoscyamus muticus* | *Rhizoctonia solani* | Accumulation of sesquiterpene phytoalexin | [123] |
| *Hyoscyamus muticus* | *Rhizoctonia solani* | Accumulation of solavetivone | [124], [125] |
| *Hyoscyamus muticus* | *Rhizoctonia solani* | Accumulation of solavetivone and lubimin | [126] |
| *Hyoscyamus muticus* | *Inonotus obliquus* | Stimulation of hyoscyamine | [8] |
| *Hyoscyamus muticus* | $CuSO_4$ | Stimulation of hyoscyamine | [8] |
| *Hyoscyamus muticus* | Purifield chitosan | Stimulation of *l*-hyoscyamine | [46] |
| *Hyoscyamus muticus* | Jasmonic acid<br>Methyl jasmonate | Slight stimulation of hyoscyamine. Strong stimulation of polyamines | [127] |
| *Hyoscyamus muticus* | Methyl jasmonate + wounding + *Rhizoctonia solani* | Solavetivone and lubimin accumulation | [128] |
| *Nicotiana tabacum* | Yeast extract, *Botrytis fabae* extract | Accumulation of sesquiterpene phytoalexins (capsidiol and debneyol) | [129] |

cell suspension cultures which, however, are not directly connected with hairy roots. This polycationic polysaccharide induces pore formation in the plasmalemma of the cell cultures of *Chenopodium rubrum*. It has been suggested that pore formation is related to the degree of the deacetylation (positive charges) of the chitosan. Consequently, highly charged chitosan polymers induce a higher degree of pore formation and cause faster secondary product release than the less charged ones. This means that there is a critical charge density, which leads to loss of cell viability. Unfortunately, most of the permeabilization agents are not, like chitosan, membrane-specific [87].

## Large-Scale Cultivation of Transformed Roots

Much work has been carried out with bioreactors and process development during the last decades. Design of the mixing system for bioreactors has been the most problematic. Mechanical agitation is seldom suitable for hairy roots because they are susceptible to shear stress that causes disorganisation and callus formation, with consequently lowered productivity.

Conventional stirred-tank reactors have been successfully applied to hairy roots even though the mixing system of such biorectors has been reported to cause shear damage [88]. However, this is the only reported study in the literature, where hairy roots were successfully cultivated in a simple stirred reactor. It seems to be clear that standard reactors are not suitable for hairy root cultures.

However, the best growth characteristics were obtained with bioreactors without mechanical stirring. The use of airlift reactors makes it possible to avoid shear stress completely, and up to 13-litre vessels have been used for the growth of hairy roots [89]. Wilson and co-workers [7], [90] have described so called droplet reactors in which the medium is sprayed over the roots and periodically sucked out, the roots being in contact with the air for most of the time. The most promising bioreactors for the cultivation of hairy roots seem to be so-called wave reactors. This reactor system has three components: a rocker unit, the disposable bioreactor chamber, and the measuring and control units. The wave reactor is a mechanically driven reactor system. The energy input is caused by rocking the chamber forth and back putting the cell culture and the medium in a wave movement. This reactor has been demonstrated to increase the growth of hairy root cultures producing tropane alkaloids and ginsenosides significantly more than optimised stirred reactors, rotating drum reactors and droplet phase reactors [91]. Pilot-scale studies with wave reactors are currently running up to 100 litre working volume (R. Eibl, personal communication). More comprehensive review on large-scale cultivation of hairy roots is presented by Eibl and Eibl [92].

## Genetic Engineering as a Tool to Increase Alkaloid Production

Metabolic engineering has been successful in micro-organisms for the increased production of pharmaceuticals and for the production of new compounds, for instance antibiotics. Although efficient methods for gene cloning, including organ-specific promoters, transformation (e.g., particle bombardment and *Agrobacterium*-mediated gene transfer), and regeneration of transgenic plants, are available, the progress to improve medicinal plants has so far been relatively slow. This is due to the fact that still very little is known about the biosynthesis of secondary metabolites. Often the biosynthetic routes are very long and complicated requiring several steps and key enzymes before the desired end product is formed [4]. The majority of plant genes involved in primary metabolism have now been identified due to large-scale DNA sequencing projects whereas only very limited numbers of genes involved in secondary metabolism are available.

Most enzymes in a given pathway are co-ordinately regulated, and it is speculated that there are, in the case of secondary metabolites, no clear rate-limiting enzymes as is the case for primary metabolism. Catalytic activities of individual enzymes in a pathway often vary considerably, which may result in accumulation of some intermediates unless metabolic channelling or compartmentation occurs. Pathways are controlled by the cellular development but also partly induced by exogenous and endogenous signals. One major limitation to modifying an existing biosynthetic pathway by introducing a foreign enzyme is the substrate specificity because the enzyme must act on an intermediate in that specific pathway. The regulation of enzyme levels and activity is the most important factor in the control of the production of pharmaceutical compounds [2], [93].

Different approaches can be applied for obtaining higher yields of a desired compound in transgenic plants or cell cultures: engineering single biosynthetic steps, mapping regulatory genes, reducing flux towards competitive pathways and/or catabolism, and increasing the number of producing cells [93]. Several reporter genes, such as neomycin phosphotransferase II (*kan*) and $\beta$-glucuronidase (*gus*), have been used as models in investigating gene expression in several medicinal plants of interest [94], [95]. Although reporter genes were not expected to have an effect on secondary metabolite production, there are a few examples where the formation of secondary metabolites has either been stimulated or inhibited by the reporter gene [94], [96].

In recent years characterisation of the enzymes involved in the biosynthesis of alkaloids has increased exponentially [3]. From a pharmaceutical point of view, the first successful example of engineering a medicinal plant was performed by Yun and co-workers [97]. They cloned the hyoscyamine 6$\beta$-hydroxylase gene (*h6h*) of *H. niger* and introduced it to *A. belladonna*, which produces hyoscyamine as the main alkaloid and very little scopolamine. The engineered *A. belladonna* hairy roots exhibited increased hydroxylase activity and produced five-fold higher concentrations of scopolamine than the wild-type hairy roots [98]. The transgenic *A. belladonna* plants almost exclusively produced scopolamine [97]. The same gene alone or in combination with other regulatory genes has been further transferred to different tropane alkaloid-producing plant species, and the effects on secondary metabolism vary considerably. Transgenic root cultures of *H. muticus* carrying the *h6h* transgene were able to produce over 100 times more scopolamine than the control ones [99]. Interestingly in this study also the hyoscyamine levels remained high contrary to the findings in *A. belladonna*.

Besides the effective research carried out on the tropane alkaloid pathway [100], a lot of effort has been put in the understanding of regulation of indole alkaloid (particularly vincristine and vinblastine, two high-value anticancer drugs) production in *C. roseus*. These investigations, however, have so far had only limited success [101]. It might be due to the fact that the introduced enzymes do not catalyse critical rate-limiting steps in the target biosynthetic pathways [98].

## Conclusions

Despite the promising features and developments, the production of plant-derived pharmaceuticals by hairy roots has not yet been commercially exploited. The main reasons for this reluctance shown by industry to produce pharmaceuticals by means of hairy roots compared to the conventional extraction of whole plant material are mainly economical ones based on the too low contents. Furthermore, the compound produced by this novel production system has to be re-evaluated by authorities for quality, efficacy and safety reasons which might inhibit the industry for using this technology. However, we are convinced of the rapid development of genomics, proteomics and metabolomics tools will create great opportunities to engineer the often complex pathways of plant secondary metabolites and thus increase the contents of high-value pharmaceuticals in plant cell or organ cultures.

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