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Agrobacterium rhizogenes-Mediated Transformation of Medicinal Plants from the Family *Rhamnaceae*

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ABSTRACT

A number of plants can be successfully transformed by *Agrobacterium rhizogenes* through the transfer of T-DNA from agrobacteria to the plant genome. Transgenic tissue – hairy roots – is produced as a result of the transformation process. This organized, genetically stable, hormone-independent transformed tissue is capable of accomplishing complex metabolic pathways, including biosynthesis and accumulation of various secondary metabolites. Somaclonal variation is often observed among the hairy root cultures. The highly-productive hairy root lines, containing a large amount of important metabolites can be selected and grown *in vitro* on hormone-free media for a long period of time, whilst preserving their biosynthetic capacities. Consequently, during the last decade, hairy root cultures have been recognized as an excellent system for *in vitro* generation of a large biomass of transgenic tissue that could be utilized for the extraction of desired metabolites or even in the development of new compounds through novel metabolic pathways. Species from the family *Rhamnaceae* are well known for their capacity to synthesize the aromatic carbohydrates, anthraquinones (AQs). These metabolites with laxative action are traditionally extracted from the bark of *Frangulae* cortex. Applying a genetic engineering approach, the hairy root cultures of *Rhamnus fallax* open a convenient alternative for the production of increased amount of medically important metabolites such as AQs while protecting natural recourses and environment.

Keywords: anthraquinones, genetic transformation, hairy roots, *Rhamnus*, secondary metabolites
Abbreviations: AQs, anthraquinones; **GM**, genetically modified; **MS**, Murashige and Skoog's (1962) medium **SM**, secondary metabolites

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INTRODUCTION

Plant biotechnology is a modern tool of plant breeding, where novel traits are introduced by genetic transfer to plant genome producing as a result transgenic plants or tissue. After generating first genetically modified plant more then 20 years ago, plant biotechnology began its golden period. Transgenic plants are obtained with a range of desired, new or improved features such as resistance to diseases, increased tolerance to herbicides and pesticides, as well as with enhanced content of desired compounds. For decade, transgenic crops have been utilized and commercially grown worldwide. Rough estimation of total approved areas occupied by genetically modified (GM) crops reached 90 million hectares in 2005 (http://www.isaaa.org/kc/bin/briefs34/es/index.htm). Globally grown transgenic crops include soybean (60% of area occupied by GM crops), maize (24%), cotton (11%) and canola (5%), with majority (more than 50%) developed in USA. GM crops excepted for breeding in the field are characterized with herbicides and pesticide resistance traits (Wenzel 2006).

One of the most common ways to transfer foreign gene(s) to the plant genome is using *Agrobacterium*, natural biological vector gram-negative soil bacterium. The *Agrobacterium*-mediated transformation results in production of transformed tissue "crown gall tumors" when infected by *Agrobacterium tumefaciens* or "hairy roots" in the case of *A. rhizogenes* (Tepfer and Casse-Delbart 1987; Constabel 1990). A high stability of transgenic root cultures and their fast growing can be efficiently utilized in large scale bioreactors (Wilson 1997). Consequently, hairy root cultures carry out a great potential for the synthesis and mass production of secondary metabolites (Guillon *et al*. 2006a). Plant secondary metabolites (SM) are a huge group of compounds with significant importance in ecology, food production and pharmaceutical industry (Acamovic and Brooker 2005). Some medicinal plants are used as a natural source of these phytochemicals. Plants from the family *Rhamnaceae* have medicinal importance, which have been recognized due to a high content of anthraquinones (AQs)

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(Rosic *et al*. 2000, 2006). AQ compounds are well-known for their laxative action, while *Rhamnaceae* species have been used as a natural source of these medicinally important SMs.

Indeed, protection of the environment and natural resources, especially when endanger species are the only source of pharmaceutical compounds, outlined the necessity for alternative solutions such as plant engineering and hairy root cultures. The aims of this review are to in general highlight advantages and disadvantages of *Agrobacterium*-mediated transformation; to give overview of current knowledge of hairy root cultures and its application in medicinal plants from the family *Rhamnaceae* in the production of AQ secondary metabolites.

MEDICINAL PLANTS

For a long period of time, plants have been used to provide people shielter, food, clothing, as well as medicines (Gurib-Fakim 2006). In ancient cultures, traditional medicines were empirically developed and verbally passed through generations. Nowadays, using old knowledge of ethnobotany and ethnopharmacognosy, there is a search for "new" recourses of medicinal compounds, which could be potentially utilized in pharmaceutical industry. The other, modern approach uses combinatorial biosynthesis for the generation of novel compounds. In combinatorial biosynthesis SMs are produced in the heterologous expression system, combining known metabolic pathways from different organisms (Julsing *et al*. 2006). During the last two decades molecular plant breeding has been frequently used as another modern tool for the production of medicinally valuable compounds. For the mass production of these desired metabolites, hairy root cultures are often recognized as a good and stable system (Guillon *et al*. 2006a, 2006b). Using these novel tools, combinatorial biosynthesis and plant biotechnology, open possibilities to produce compounds that could not be extracted from natural recourses due to a small concentration present in plants tissue or due to small quantity of these species in nature.

Plants from family *Rhamanceae*

Plants from the family *Rhamnaceae* belong to the order *Rhamnales*, class *Magnoliatae*. This plant family has more than 500 species distributed mostly in tropical and subtropical areas (Tutin 1972). *Rhamnaceae* spp. produce active SMs, AQs, which are used as a laxative. These SMs have been recognized for their laxative action from the 19th century (van Gorkom *et al*. 1999). The *Frangulae* cortex from *Rhamnus frangula* is used as an official source of AQ compounds (in Pharm.Yug. IV). However, *Fallacis cortex* obtained from *Rhamnus fallax* with a high content of AQs, is often used as a substitute, instead of the official drug (Rosic 2000). In addition, as natural sources of anthranoid laxatives are also used dried parts of *Cassia acutifolia* and *C. angustifolia* (famous for a high content of AQs – sennosides) cultivated mainly in India and Egypt, then *Rhamnus purshiana* and *Rheum palmatum* (van Gorkom *et al*. 1999). These AQ drugs are used in traditional medicine in the form of tea (*Species laxantes*).

The AQ compounds belong to the group of hinone pigments, which are mostly yellow colored pigments. AQs contain the basic structure made from 9,10-dioxoanthracene (**Fig. 1**). In man, the laxative action of AQs is a result of the activity of their sugar derivates (glycosides). In the colon, reduced forms, anthrones and dianthrones, have a laxative action and they are released in an enzymatic reaction catalyzed by bacterial enzymes. This results in changes on the colonic epithelium, in colonic absorption and colonic motility, resulting in fluid retention. Except for their laxative action, AQ drugs have antimicrobial and antiseptic properties, and therefore they are also utilized in treatment of different skin conditions and in dermatology (Lukic 1985).

There are two major types of glycosides found in plants,

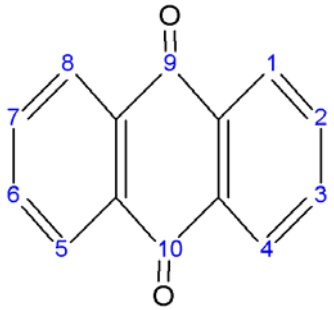

Fig. 1 The molecule 9,10-dioxoanthracene constitutes the basic structure for antraquinone derivatives, which are present in plants.

O-glycosides, containing glucose or rhamnose bound to the OH group of the C-8 atom and C- glycosides, with a sugar attached directly to the C-10 atom (Lukic 1985; van Gorkom *et al*. 1999). The major fractions of AQ compounds found in different tissues of *R. fallax* collected from nature included: aloe emodin, emodin, chrysophanol and phiscion (**Table 1**) (Rosic *et al*. 2000). Similar predominance of AQ composition of aloe emodin, emodin, chrysophanol and phiscion was also revealed for *in vitro* cultures of callus and shoots.

Table 1 The mean values for AQ compositions in the different tissues of *R. fallax* collected in nature and from culture (based on Rosic *et al*. 2000).

Tissue		Ratio of main AQ compounds			
		aloe emodin	emodin	chrysophanol	phiscion
Nature	leaves	17	41	31	11
	bark	4	34	8	54
	unripe fruit	62	22	15	1
	ripe fruit	77	12	9	2
Culture	shoot	19	19	29	2
	callus tissue	18	42	19	21

PLANT TRANSFORMATION

Different approaches in plant engineering

Nowadays, using genetic engineering techniques foreign genes can be introduced into any living organism, resulting in the generation of genetically modified organisms (GMOs). The methods of plant genetic engineering allow direct or indirect gene transfer into the plant genome. There are many different methods used for the direct gene transfer such as the PEG (polyethylene glycol) method, where DNA transfer is chemically facilitated into the protoplast; electroporation, which uses an electrical field to speed up the DNA transfer into protoplast; biolistics and microinjection. The indirect gene transfer methods include the use of biological vectors such as *Agrobacterium* spp. These methods are broadly used methods, being highly efficient and cost-effective compared to other methods of plant transformation (Vergunst and Hooykaas 1999).

Vegetative propagation of plants from the family *Rhamnaceae* (*R. fallax*, *R. orbiculatus*, *Frangula rupestris* and *F. alnus*) has been highly successful in *in vitro* culture (**Fig. 2**). Optimal media composition and hormonal content applicable for different *Rhamnaceae* spp. includes Murashige and Skoog (1962; MS) basal medium containing MS mineral salts and vitamins, as well as 0.5 µM indole-3-butyric acid (IBA) and 5 µM 6-benzylaminopurine (BAP) as reported previously for *R. fallax* (Rosic *et al.* 2000). The effective propagation of *Rhamnaceae* plants *in vitro* opened the possibility for their exploitation and manipulation by genetic engeenering.

Agrobacterium-mediated transformation

Within the family *Rhizobiaceae*, gram-negative soil bacteria in the genus *Agrobacterium* includes all virulent species, with the exception of one non-virulent species, *A. radiobacter* (Depicker *et al*. 1983). Virulent types of *Agrobacterium* are capable of infecting plants resulting in the development

Fig. 2 *Rhamnus fallax* obtained from culture *in vitro*.

of tumor cells at the infection site. There are two types of transformed tissue that develop on the wounded plant, "crown galls" or "hairy roots" when plant cells are infected by *A. tumefaciens* or *A. rhizogenes*, respectively (Gaudin *et al*. 1994). This transformed tissue does not require plant hormones for growth when in culture (Grierson and Convey 1988). It is interesting to note that the presence of opines in tumor tissue is one of the secure signs of agroinfection. Opines are not naturally found in plants, and they are used by *Agrobacterium* strains as a source of nitrogen and carbon (Kim and Farrand 1998).

The first study of successful genetic transformation of *Rhamnaceae* plants was recently reported for *R. fallax*, by *A. rhizogenes* (Rosic *et al*. 2006). In this review, for the fist time I present the results of genetic transformation of three other members of the *Rhamnaceae* family: *R. orbiculatus*, *Frangula rupestris* and *F. alnus* by *A. rhizogenes* (strain A4M70GUS), using two approaches: stabbing of the needle infected with bacterial suspension) into the second node and dipping the cut petioles into bacterial suspension. Positive sign of infection was visualized by appearance of callus tissue and hairy roots at the infected site. Higher transformation efficiency for all *Rhamnaceae* spp., with up to 50% infected samples (50 of 100 inoculated samples), was obtained with the stabbing approach, while quite lower efficiency (up to 27%) was revealed by dipping of petioles into bacterial suspension. Transformation of four *Rhamnaceae* spp. when using *A. tumefaciens* (strain A_{281}/ pTiBO542) resulted in the appearance of "crown galls" at the site of infection, with again a higher efficiency obtained by stabbing bacterial suspension into plants (up to 28% of infected samples). Intensive cell division in nodal area of stems might facilitate agrobacteria infection and increase chances for *Rhamnaceae* spp. to be genetically modified.

The transformed tissue, hairy roots and callus cultures, of all four *Rhamnaceae* were stable in culture *in vitro* for more then several years, preserving their transgenic features, such as hormone independence and stable growth over the period of time. However, there were no signs of spontaneous regeneration on transformed roots and callus. In addition, serious attempts to initiate plant regeneration with a range of different cytokinins and auxins, as well as various amino acids have failed (data not shown). Consequently, future studies should try to bring more understanding behind deprived capacity for plant regeneration observed in transformed tissue of analyzed species from the *Rhamnaceae* family.

Hairy roots: characteristics

The *A. rhizogenes* strains carrying the *Ri*-(root inducing) plasmid result in the manifestation of "hairy roots" disease at the place of infection (Hooykaas and Schilperoort 1992). The development of hairy roots is the result of transfer of *A. rhizogenes* DNA segment (T-DNA) into plant genome. After infection, genes carried by the T-DNA change the hormonal equilibrium in the infected plant cells by affecting the biosynthesis of plant hormones auxin and cytokinin. In general, hairy root cultures are well characterized in their phenotypic features, which include rapid growth, reduced apical dominance, lack of geotropism and preserved biosynthetic capacity for the production of SMs. Since many interesting SMs accumulate in roots, hairy roots cultures have been accepted as a good alternative for the *in vitro* production of valuable metabolites (Giri and Narasu 2000; Guillon *et al*. 2006a). In the last couple of years there has been significant progress in the use of hairy root cultures for the production of active SMs from medicinal plants as recently reviewed by Guillon *et al*. (2006a, 2006b).

Our team recently reported that AQ compounds can be produced from hairy root cultures (Rosic *et al*. 2006). The medicinal plant *Rhamnus fallax*, from the *Rhamnaceae* family, was infected with *A. rhizogenes* and a stable culture of transformed roots was obtained. The hairy root cultures were characterized with two morphological types (**Fig. 3**). One root-culture type contained white colored roots and more root hairs than the second type, which was further distinguished by yellow roots and more lateral branching. Both morphological types preserved their natural capacities towards biosynthesis of AQ compounds. Yet yellow colored clones contained a higher level of AQs when compared to other analyzed material from nature and culture. The AQ content of 16.43 mg and 14.21 mg/g of dry mass were quantified in yellow colored clones 1 and 7, respectively (Rosic *et al.* 2006). The richest part of *R. falax* tissue collected in nature, bark and unripe fruit, contained 13.50 mg and 13.60 mg of AQs, respectively (Rosic 2000). The basis for an increase in the AQ content observed in clones 1 and 7 is yet to be assessed. Hence, improved capacity for SMs synthesis in transgenic roots with altered morphological characteristics might be the result of a change in their metabolic pathways after insertion of T-DNA into the plant genome (Moyano *et al*. 2003).

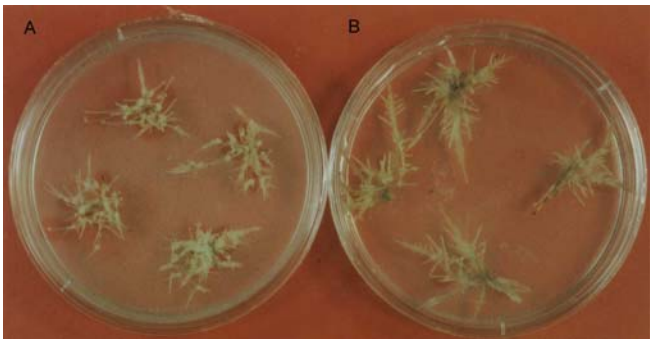

Fig. 3 Hairy root cultures of *R. fallax*. Two different morphological types were distinguished: (**A**) white roots with a vast number of root hairs and (**B**) yellow roots, with a large number of lateral roots, but not as many root hairs as type A. Adapted from Rosic *et al*. (2006).

CONCLUSIONS

The hairy root cultures with preserved biosynthetic capacities keep providing more and more supporting evidence for biotechnologists with respect to their genetic stability in cultures *in vitro* over a long period. Consequently, the list of active secondary metabolites that can be extracted from transformed roots inevitably gets longer with time. The AQ compounds as well-known laxatives have been recently in-

cluded on the list of valuable metabolites produced by hairy root cultures (Rosic *et al*. 2006). Future studies should go a step further in merging the production of SMs from hairy root cultures with pharmaceutical requirements. Hence, ecological aspects aiming to preserve natural diversity and global environment should be met in the near future.

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