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# Genetic transformation of *Rhamnus fallax* and hairy roots as a source of anthraquinones

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## Abstract

Hairy roots of *Rhamnus fallax* Boiss. were induced using *Agrobacterium rhizogenes* strain A4M70GUS. The culture established on Woody plant media (WPM) showed a typical hairy root phenotype: rapid growth, reduced apical dominance and root plagiotropism. Seven clones of *R. fallax* were selected on the basis of their differences in colour and the root branching. The growth of hairy root culture, measured through gain in fresh mass, was done under 16-h photoperiod or in the dark. An increase in anthraquinone (AQ) content was obtained in clones with yellow and less branched roots, like clone 1 [16.43 mg $g^{-1}$(d.m.)] and clone 7 [14.21 mg $g^{-1}$(d.m.)], compared with other analysed transformed and non-transformed tissue. This study presents the first report of successful transformation of any species from family *Rhamnaceae* by *A. rhizogenes* and analysis of AQ production in transformed tissue.

*Additional key words*: *Agrobacterium rhizogenes*, transformed roots, secondary metabolites.

## Introduction

Species from the family *Rhamnaceae* produce anthraquinones (AQs), active secondary metabolites, which have medicinal importance. *Rhamnus fallax* Boiss. is a shrubby perennial plant growing in the mountain regions of Europe. This shrub is often used as substitute for official drug *Frangulae cortex* in Pharm. Yug. IV. The protection of an environment enlarged demands in finding alternative sources for mass production of this drug. One of the most appropriate approaches for increasing production of many pharmaceutically important compounds is the application of genetically transformed roots.

*Agrobacterium rhizogenes*-mediated transformation of plants is applicable to many dicotyledonous and several monocotyledonous plant species. *A. rhizogenes* with Ri-(root inducing) plasmid induces the appearance of the "hairy roots" disease, due to the introduction one or both of the two DNA pieces ($T_L$ and $T_R$-DNA), their integration and expression of bacterial oncogenes into the plant genome (Hooykaas and Schilperoort 1992). Since the genetic transformation does not impair the natural root synthetic capacities, hairy roots have been recognized as potential alternative source of many secondary compounds with comparable or even greater level of metabolites production than in the intact plants (Giri and Narasu 2000). Fast growing and genetically stable, hairy roots can be efficiently cultured in large scale bioreactors (Wilson 1997).

In the investigation reported, we aimed to obtain hairy root cultures of *R. fallax*, to optimize the condition for their growth and to assess AQs production in transformed tissue.

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*Abbreviations*: AQ - anthraquinone; BAP - 6-benzylaminopurine; GB5 medium - Gamborg B5 medium; IBA - indole-3-butyric acid; MS - Murashige and Skoog's (1962) medium; NN medium - Nitsch and Nitsch medium; PCR - polymerase chain reaction; WPM - Woody plant medium; YEB - yeast extract broth.
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## Materials and methods

**Plants:** Vegetative propagation of *Rhamnus fallax* Boiss. (Rosić *et al.* 2000) was carried on Murashige and Skoog (1962; MS) basal medium containing MS mineral salts and vitamins, 0.7 % (m/v) *Bacto-agar*, 3 % (m/v) sucrose, 100 mg $dm^{-3}$ myo-inositol, 0.5 μM indole-3-butyric acid (IBA) and 5 μM 6-benzylaminopurine (BAP). The pH of the media was adjusted to 5.8 prior to autoclaving at 114 °C for 25 min.

**Bacterial culture:** The agropin strain of *Agrobacterium rhizogenes* A4M70GUS, constructed by Dr. M. Tepfer (INRA, Versailles, France) was used for this work. This strain is harbouring cointegrative plasmid with GUS construct integrated into $T_L$ region of pRiA4 (Tepfer and Casse-Delbart 1987). GUS construct contains *uidA* sequence under the enhancer-doubled 35S CaMV promoter. Bacterial strain was maintained on yeast extract broth (YEB) nutrient medium (Van Larebeke *et al.* 1977) solidified with 1.5 % (m/v) agar and supplemented with 100 mg $dm^{-3}$ neomycin sulfate (*ICN Biomedicals Inc*., New York, USA). Bacterial suspensions were incubated with shaking at 220 rpm for 24 h prior to inoculation, at 28 °C. Suspension at a density of $10^8$ cells $cm^{-3}$ was used for inoculation.

**Genetic transformation** was preformed on stem cuttings 3 - 4 cm long (5 internodes), propagated *in vitro* as described above. Under aseptic conditions, two methods of inoculation were used: dipping cut petioles into bacterial suspension and stabbing the infected needle into area of second node. After 48 h of incubation, the elimination of *A. rhizogenes* was done by transferring the wounded shoots to solidified MS basal media supplemented with antibiotic (300 mg $dm^{-3}$ cefotaxime, *Jugoremedia*, Zrenjanin, Serbia and Montenegro). After two weeks, adventitious roots were developed and their tips were excised and transferred on Woody plant (WPM) solid media (Lloyd and McCown 1980) with half-strength of macronutrients and supplemented with 300 mg $dm^{-3}$ cefotaxime.

In addition, for assessing the effect of different macronutrients on growth of hairy roots culture on MS, Nitsch and Nitsch (1969; NN) and Gamborg *et al.* (1968; GB5) media were used, except WPM medium. All cultures were maintained in a controlled environment room at 25 ± 2 °C, using cool white fluorescent light (irradiance of 47 μmol $m^{-2}$ $s^{-1}$) under a 16-h photoperiod. The gain in fresh mass was measured after 28 d for root cultures grown on solid and liquid media, in dark or 16-h photoperiod.

**Evidence for transformation**: The modified hexadecyltrimethylammonium bromide (CTAB) procedure was used for DNA extraction from transgenic roots and leaves of non-transformed plants (Sul and Korban 1996). PCR analysis of genomic DNA was based on the standard protocol of *Perkin Elmer* (*Roche Molecular Systems*, Brancherburg, USA). The primers used for amplification of the *aux1* sequence (656 bp) from $T_R$-DNA were, forward ($A_1$) 5′-CTCAAGAGCGCTACTCCTT CAAGTG-3′ and reverse ($A_1$) 5′-TCTCCCGCTTTC CAGATATATTGAC-3′ ($A_2$). For amplifications of *rolC* sequence ($T_L$-DNA) size 278 bp, were used primers: forward ($O_{12+}$) 5'-CCACGGGCTGCTGTACTTCTAC-3' and reverse ($O_{12-}$) 5'-TTTCCCTTTGTCGAAGTTAGC TCC-3'. In the first PCR cycle, the samples were heated to 95 °C for 4 min. This was followed by 30 cycles at 94 °C for 30 s, 55 °C (for *aux1*) or 60 °C (for *rolC*) for 30 s, 72 °C for 45 s; and the last cycle of 72 °C for 5 min (*Genius* DNA Thermal Cycler-*Techne*, Cambridge, UK). Amplified DNA was analyzed on 1.5 % (m/v) agarose gels. Plasmid DNA from *A. rhizogenes* A4M70GUS was used as a positive control and DNA from leave of non-transformed plants as a negative control.

**Qualitative and quantitative analysis of AQs**: The Bornträger test was used for identification of free AQs and *O*-glycosides of AQs in hairy roots, while *C*-glycosides of AQs were analyzed by two-dimensional thin layer chromatography (TLC) using direct acid hydrolysis on the plate. The quantity of AQs was determinate using HPLC. Extraction of AQs from plant material was performed according to a modified procedure of Van der Berg (Van der Berg and Labadie 1984), which permits the analysis of both free AQs and AQs liberated from *O*-glycosides after acid hydrolysis. Dried and pulverized samples of hairy roots (0.2 g) were extracted with ethyl ether (50 $cm^3$) under reflux for 15 min. The ether solution was dried over anhydrous sodium sulphate and ether evaporated in vacuum (30 °C) to obtain the dry residue containing free AQs (fraction A). AQ glycosides in the solid residue, remaining after the extraction of free AQs, were subjected to hydrolysis and oxidation by boiling in 50 $cm^3$ of 2 M HCl with addition of 1 g of $FeCl_3$ under reflux for 60 min. After cooling and filtration, the filtrate and macerate were extracted with ether (5 × 25 $cm^3$). Combined ether fractions were washed with distilled water (3 × 30 $cm^3$) and dried over anhydrous sodium sulphate. Ether was evaporated in vacuum (30 °C) and the dry residue containing AQs liberated from O-glycosides (fraction B). Fraction A and B were dissolved in 1 $cm^3$ of methanol and subjected to HPLC analyses [*Varian 9060-Polychrom*, UV detector, *Lichrosphera*$^R$ column 100: RP-18 (5 μm)] under following conditions: mobile phase was methanol + 1 % acetic acid in water (80:20); flow rate was 1 $cm^3$ $min^{-1}$; temperature of column was 40 °C; injection volume was 0.01 $cm^3$ and acquisition of spectra was done at 362 nm. Identification of the main chromatographic peaks obtained from analysed fraction was done by comparison their retention times and UV

spectra with those of authentic compound (Emodine and Chrysophanol – *Fluka AG*, Buchs, Switzerland; Aloe-emodine and Physcione – *Carl Roth KG*, Karlsruhe, Germany). Quantification was done by an external calibration method.

## Results and discussion

The appearance of roots at the site of inoculation was the first signs of successful infection by *A. rhizogenes* A4M70GUS. The transformation by dipping cut petioles into bacterial suspension gave only 10 % of infected samples compared to 50 % achieved by stabbing the infected needle into area of second node. Several studies showed puncturing the stems with needle loaded with bacteria as an efficient way for successful transformation by *A. rhizogenes* (Subotić *et al.* 2003/4, Bjelović *et al.* 2004). Also, the differences in the transformation efficiency depended on the transformation procedure, as well as *Agrobacterium* strain, plant species, hormonal state of the tissue, was also reported by Gaudin *et al.* (1994). Excised root tips were sub-cultured on agar-solidified WPM hormone free medium, supplemented with 300 mg $dm^{-3}$ cefotaxime. Each root tip was treated as a separate clone. All of the clones exhibited the phenotypic characteristics of transformed roots: rapid growth, hormone autotrophy, reduced apical dominance, high branching and root plagiotropism. Also, the root cultures of *R. fallax* were established on MS, NN and GB5 media, but low multiplication rate and significant presence of necrotic tissue compared with cultures obtained on WPM medium was observed. Therefore, WPM medium was used for conducting further experiments. The beneficial effect of WPM macronutrients on the growth of transformed roots of *R. fallax* may be due to a lower content of $NH_4^+$ and $NO_3^-$ ions, than in media with MS, GB5 and NN mineral salts (Wysokinska and Rózga 1998). After several subcultures on hormone free WPM medium, two types of root culture morphology were observed. Clones 2, 3, 4, 5, 6 (Fig. 1*A*) were distinguished by white roots with a vast number of root hairs (typical hairy root phenotype) and clones 1 and 7 were characterized by a yellow root colour, a large number of lateral roots and less number of root hairs (Fig. 1*B*). The estimation of the growth capacity for different clones was done by measuring a gain in fresh mass of transformed roots grown on solid and liquid WPM media, under 16-h photoperiod or dark, after 28 d (Table 1). Interestingly, the light increased gain in mass in most of the clones and even up to 4 - 5 folds in clones 1 and 5. Transformed tissues were characterized by substantial variation in growth, probably due to independent transformation event each clone was raised from (Moyano *et al.* 1999).

Molecular evidence for successful transformation was obtained by PCR analysis using primers for *rolC* (278 bp) and *aux1* (656 bp) sequence, in hairy root clones 1, 6 and 7 (Fig. 2). The presence of *rolC* sequence from $T_L$-DNA was confirmed in all analyzed clones of *R. fallax* (Fig. 2, lines 3 - 5), as well as *aux1* sequence from $T_R$-DNA (Fig. 2, lines 9 and 11), except in clone 7 (Fig. 2, line 10). These results confirmed independent insertion of $T_L$ and $T_R$-DNA into plant genome (Bundock and Hooykaas 1998). Also, it is well known that $T_R$-DNA is not essential for hairy root phenotype, but that *aux1* gene from this segment provides an additional source of auxin

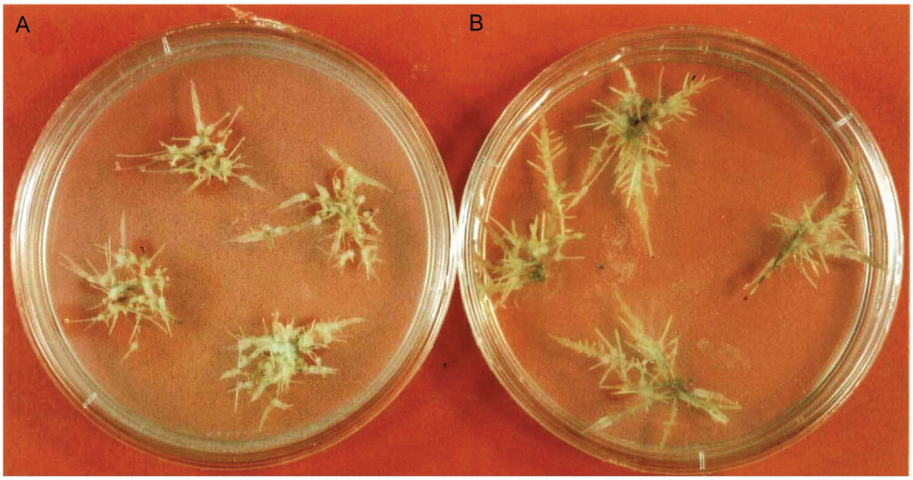


Fig. 1. The hairy-root cultures of *R. fallax* grown on half-strength WPM media (28 d, 16-h photoperiod). *A*: the root culture of clone 5 represents first morphological type, characterized by appearance of white coloured roots and a vast number of root hairs. *B*: the root culture of clone 7 corresponds to second morphological type distinguished by yellow colour, large number of lateral roots and less number of root hairs.

Table 1. AQs production (content of total AQs and ratio between free AQs and AQs liberated from *O*-glycosides [%]) in different clones of *R. fallax* transformed roots grown on solid media under 16-h photoperiod or in the dark, for a period of 28 d. The growth capacity of all clones, grown on solid or liquid media, was estimated through measuring a gain in fresh mass (means ± SE, $n = 3$).

| Clones | Cultivation | Total AQs [mg $g^{-1}$ (d.m.)] | Ratio [%] | Gain in f.m. (solid media) [g] | Gain in f.m. (liquid media) [g] |
|---|---|---|---|---|---|
| 1 | light | 7.68 | 97/ 3 | 0.929 ± 0.010 | 3.981 ± 0.162 |
| | dark | 16.43 | 88/12 | 0.391 ± 0.051 | 0.758 ± 0.049 |
| 2 | light | 4.85 | 73/27 | 0.590 ± 0.292 | 1.210 ± 0.315 |
| | dark | 3.72 | 54/46 | 0.177 ± 0.016 | 0.479 ± 0.162 |
| 3 | light | 8.52 | 73/27 | 0.506 ± 0.048 | 0.212 ± 0.056 |
| | dark | 9.47 | 73/27 | 0.452 ± 0.024 | 0.359 ± 0.043 |
| 4 | light | 4.01 | 36/64 | 1.314 ± 0.010 | 0.892 ± 0.215 |
| | dark | 3.34 | 90/10 | 1.066 ± 0.132 | 0.933 ± 0.246 |
| 5 | light | 2.80 | 50/50 | 0.692 ± 0.032 | 4.109 ± 0.664 |
| | dark | 5.55 | 95/ 5 | 0.922 ± 0.194 | 0.641 ± 0.023 |
| 6 | light | 4.28 | - | 1.172 ± 0.066 | 3.373 ± 0.750 |
| | dark | - | - | 0.443 ± 0.055 | 3.960 ± 0.887 |
| 7 | light | 14.21 | - | 1.433 ± 0.175 | 0.276 ± 0.055 |
| | dark | - | - | 0.879 ± 0.065 | 0.362 ± 0.033 |

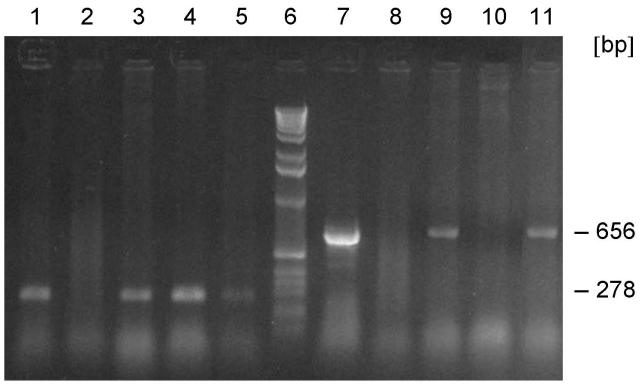


Fig. 2. Electrophoretic separation of the PCR products obtained by $O_{12+}/O_{12-}$ primers for *aux1* sequence (656 bp) from $T_R$-DNA in *lines 1 - 5* and by $A_1/A_2$ primers *in lines 7 - 11* for a presence of *rolC* sequence ($T_L$-DNA) size 278 bp. *Lines 1* and *7* - DNA from $A_4$ plasmid as a positive control; *lines 2* and *8* - DNA from leaves of non-transformed plants as a negative control; *lines 3* and *9* - DNA from clone 6; *lines 4* and *10* - DNA from clone 7; *lines 5* and *11* - DNA from clone 1; *line 6* - DNA ladder.

to the transformed cells (Sevon and Oksman-Caldentey 2002). However, *rol* genes from $T_L$-DNA have the major role in induction of hairy roots formation and positive effect on secondary metabolites production in transformed tissue. For example, as result of *rol*-gene transformation significant increase of AQs content was reported in transformed callus culture of *Rubia cordifolia* (Bulgakov *et al.* 2002).

The presence of AQ compounds in the free form and in the form of *O*-glycosides was confirmed in hairy roots of *R. fallax*, while *C*-glycosides were absent or detected in very low amount (data not shown). The major fractions of AQ components in analyzed tissue were aloe-emodin, emodin, chrysophanol and physcion. The quantitative analysis showed dominant presence of free AQs in most of the analysed clones grown in 16 h day period and under darkness (Table 1). However, in the presence of light clone 5 accumulated 50 % and clone 4 even up to 64 % of glycosidically bound AQs. Similarly, AQ glycosides were found as a dominant fraction in material collected from nature and in non-transformed tissue from culture (Rosić 2000). The established culture of hairy roots preserved capacity for AQs production and the bias accumulation of free AQs what could be due to changes in metabolic pathways of transformed tissue. Also, the variation of radiation conditions, in our case a presence and absence of light, could effect the accumulation of metabolites (Shin *et al.* 2003/4). Comparative analysis of AQs production in transformed roots and their growth capacity (after 28 d, on solid media, under a 16 h-photoperiod) showed huge differences between clones (Table 1). The best promising

results regarding growth and biosynthetic capacity was observed in clone 7, with 14.21 mg(AQs) $g^{-1}$(d.m.). This clone showed the highest gain in mass, as well as the highest content of AQs compared to all analysed clones. Also, clone 1 showed high AQs production [16.43 mg $g^{-1}$(d.m.)] while growing in darkness. It is interesting to note that clones 1 and 7 had high AQs production whilst being morphologically distinct. Yellow colouration and altered phenotype of these clones, could be result of the modification of metabolic pathways and accumulation of other compounds (Moyano *et al.* 2002). These two clones overcome the AQs content in the richest parts of tissue collected from nature, as are bark and unripe fruit (Rosić 2000).

In conclusion, we report first successful way for genetic transformation of *R. fallax* using *A. rhizogenes* as a natural biological vector. The stability of hairy root cultures over couple years, as well as their preserved biosynthetic capacities is promising result for future use of genetically engineered sources with enhanced level of AQs production.

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