

# Sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum* Griseb. for bioactive metabolite accumulation

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**Abstract** An efficient protocol for the establishment of transformed root culture of *Verbascum xanthophoeniceum* using sonication-assisted *Agrobacterium rhizogenes*-mediated transformation is reported. Only 10 days after the inoculation with *A. rhizogenes* ATCC 15834 and 45 s ultrasound exposure, hairy roots appeared on 75% of the *Verbascum* leaves. Ten hairy root lines were isolated, although only half of them were free of bacterial contamination and started growing when excised from mother explants. The transgenic nature of the most vigorously growing hairy root clones (VX1 and VX6) was confirmed by polymerase chain reaction. Under submerged cultivation both hairy root clones accumulated high biomass amounts (12.8 and 14.3 g L<sup>-1</sup>, respectively) and significant amounts of bioactive phenylethanoid glycoside

verbascoside (over 6-times more than in mother plant leaves). LC-APCI-MS analyses confirmed verbascoside accumulation in hairy root clones along with three other phenylethanoid glycosides (forsythoside B, leucosceptoside B and martynoside) and an iridoid glycoside aucubin. This is the first report on the induction of hairy roots of *Verbascum* plants.

**Keywords** *Agrobacterium rhizogenes* · Hairy roots · LC-APCI-MS · Phenylethanoid glycosides · SAAT · *Verbascum xanthophoeniceum*

## Abbreviations

ADB	Accumulated dry biomass (g L <sup>-1</sup> )
CTAB	Cetyltrimethylammonium bromide
GI	Growth index
HPLC–DAD	High performance liquid chromatography with diode array detection
LC-APCI-MS	Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry
<i>m/z</i>	Mass-to-charge ratio
ORF	Open reading frames
PCR	Polymerase chain reaction
SAAT	Sonication-assisted <i>Agrobacterium</i> -mediated transformation

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## Introduction

*Verbascum* L., common name Mulleins, is a genus of 360 species of flowering plants in the Scrophulariaceae family, predominantly distributed in Asia, Europe, and North America (Heywood 1993). Mulleins are biennial or

perennial, rarely annual plants, with a deep tap root. At the first year plants form a dense rosette of leaves, which is followed by growth of a stout flowering stem 0.3–2 m tall (Turker and Gurel 2005). Several records exist for the medical use of mulleins in folk medicine as a remedy for respiratory problems such as bronchitis, dry coughs, whooping cough, tuberculosis, and asthma. The leaves, roots and the flowers possess also anodyne, antimicrobial, anti-inflammatory, and sedative properties (Turker and Gurel 2005). Although *Verbascum* plants have been used medicinally since ancient times, their popularity increased commercially in the past few years. Today, the dried leaves and flowers, swallow capsules, alcohol extracts and the flower oil of common mullein can be found in United States health stores (Turker and Gurel 2005). *Verbascum xanthophoeniceum* Griseb. is an endemic plant species for the Balkan region, as well as for Northwestern and South parts of Turkey. It produces several bioactive iridoid and phenylethanoid glycosides (Kostadinova et al., in preparation), therefore it might serve as attractive source of potent anti-inflammatory and antioxidant compounds. In a previous report we found that phenylethanoid glycosides (forsythoside B, verbascoside and leucosceptoside B) accumulating in *V. xanthophoeniceum* aerial parts possess strong antioxidant, and acetylcholine and butyrylcholine inhibitory activities (Georgiev et al. 2010).

Genetic transformations with the ‘natural genetic engineer’ *Agrobacterium rhizogenes* for hairy root culture induction have attracted considerable attention since their immense potential to produce similar (or even higher) amounts of desired compounds (compared to mother plants) was realized about 25 years ago (Georgiev et al. 2007). The genetic and biochemical stability (being theoretically initiated from a single cell) and the fast growth in media free of exogenous phytohormones offer substantial advantages of transformed roots over dedifferentiated cell cultures (Vasilev et al. 2006; Georgiev et al. 2007; Zhang et al. 2009). It was recently estimated that hairy roots of more than 450 different plant species have been induced, including a diverse host range of dicotyledonous plants (the natural hosts of *Agrobacterium*; Georgiev et al. 2008). A major drawback of *Agrobacterium*-mediated transformations is that some plant species (mainly monocotyledonous plant families) are still considered as difficult-to-transform or even non-susceptible to *Agrobacterium* infections. Trick and Finer (1997) developed a new and potentially more efficient method to deliver *Agrobacterium* to target plant tissues. This technique, called by them Sonication-assisted *Agrobacterium*-mediated transformation (SAAT), involves subjecting the explants to short periods of ultrasound in the presence of *Agrobacterium* (Trick and Finer 1997, 1998). Performed scanning electron and light microscopy observations revealed that ultrasound treatment produces small

and uniform fissures and channels throughout the plant tissue, which allows *Agrobacterium* access to internal plant tissue (Trick and Finer 1997). Furthermore, the SAAT technology has been successfully applied for *A. rhizogenes* transformation of difficult-to-transform *Papaver somniferum* plants (Le Flem-Bonhomme et al. 2004).

The aim of the present report was to develop an efficient protocol for *A. rhizogenes*-mediated transformation of *Verbascum* plants for induction of hairy roots and to show that these hairy roots are able to produce bioactive compounds. According to the best of our knowledge this is the first report for induction of hairy roots in *Verbascum* plants.

## Materials and methods

### Intact plant material

The *V. xanthophoeniceum* plants were identified and seeds were collected from Lesovo village (Yambol district, Bulgaria) by Dr. S. Bancheva (Institute of Botany, Bulgarian Academy of Sciences). A voucher specimen was deposited in the Herbarium of the Institute of Botany, Sofia, Bulgaria (SOM). *Verbascum* seeds were sowed in decontaminated soil and grown in the laboratory, at 26°C with an illumination period of 16 h light:8 h dark. After 2 months of growth *V. xanthophoeniceum* plants formed ~10 cm (in diameter) dense rosettes. Young haired leaves were cut and gently washed with tap water. Surface sterilization was performed with 70% ethanol for 10 s, followed by a second sterilization with 6% Ca(OCl)<sub>2</sub> for 6 min. Few drops of Tween 80 were added to both sterilization agents for surface tension reduction. Sterile explants were cut to ~1 cm<sup>2</sup> pieces and used for transformation experiments.

### *Agrobacterium* strains and hairy roots induction protocol

Three different *A. rhizogenes* strains were used to infect *V. xanthophoeniceum* plants: *A. rhizogenes* ATCC 15834 (a gift from Prof. Dr. Thomas Bley, TU Dresden, Germany), LBA 9402 and TR 105 (a gift from Prof. Dr. Iliana Ionkova, Medical University, Sofia, Bulgaria). All *Agrobacterium* strains were maintained on YEB agar medium (8 g L<sup>-1</sup> beef extract, 1 g L<sup>-1</sup> yeast extract, 5 g L<sup>-1</sup> sucrose, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub> and 20 g L<sup>-1</sup> agar, pH 7.0) at 26°C. Prior to transformation experiments three strains were grown overnight (16 h) in YEB medium (minus agar), on an orbital shaker (110 rpm), at 26°C. The bacterial suspensions were centrifuged at 5,000 rpm for 25 min and then the pellets were resuspended in MS medium (Murashige and Skoog 1962) to an OD<sub>600 nm</sub> between 0.6 and 0.8.

Transformations of sterile explants were performed either through co-cultivation or through direct infection methods (Georgiev et al. 2007). For the SAAT experiments ten explants ( $\sim 1 \text{ cm}^2$ ) were transferred to sterile 50 mL Falcon tubes filled out with properly diluted in MS medium *A. rhizogenes* ATCC 15834 suspension. The plastic tubes were individually placed in a sonicator and subjected to ultrasound with frequency of 35 kHz (UCI-50Raypa<sup>®</sup> ultrasonic cleaner bath; R. Espinar S.L., Barcelona, Spain). The duration of treatments was 0, 30, 45 or 60 s. After ultrasound treatment, explants were blotted on filter paper to remove excess bacteria and transferred to solid MS media. All transformation procedures were performed in dark, at 26°C. After 3 days all explants from co-cultivation, direct infection and SAAT transformations were transferred to solid MS media, supplemented with 30 g L<sup>-1</sup> sucrose, 300 mg L<sup>-1</sup> cefotaxime sodium and 5.5 g L<sup>-1</sup> Plant agar (all from Duchefa, Haarlem, The Netherlands).

#### Polymerase chain reaction analysis of transformation

Genomic DNA from two hairy root lines (VX1 and VX6) and untransformed roots of in vitro grown *Verbascum* plants was isolated following a cetyltrimethylammonium bromide (CTAB)-Protocol that was modified after Pirttilä et al. (2001). Plant material was pulverized with a mortar and pestle under liquid nitrogen and 100–300 mg were placed in Eppendorf tubes. Seven hundred microlitre CTAB buffer (without mercaptoethanol) was added to the samples, mixed and incubated under continuous stirring for 45 min at 65°C. After centrifugation for 10 min at 15,900g the supernatant was extracted with 1 volume of chloroform/isoamyl alcohol (24:1/v:v). The aqueous phase was mixed with 1/10 volume of 3 M sodium acetate (pH 5.2) and 1 volume of ice cold (–20°C) isopropyl alcohol. DNA was precipitated by incubation for 10–15 min at room temperature and centrifugation for 20 min at 4°C and 15,900g. The pellet was washed two times with ice cold ethanol (70%/v/v), air dried and dissolved in 50 µL distilled water.

Ri plasmid DNA was isolated after Hayman and Farrand (1990). Polymerase chain reaction (PCR) was performed according to Grabkowska et al. (2010) with the following modifications: 10 pM of each primer and 100 ng DNA were in the PCR mixture, elongation time was 1 min per cycle, and all primer pairs were combined in one PCR (multiplex PCR). Primers were 5'-GCT CTT GCA GTG CTA GAT TT-3' and 5'-GAA GGT GCA AGC TAC CTC TC-3' for rolB (expected size of product 423 bp), 5'-CTC CTG ACA TCA AAC TCG TC-3' and 5'-TGC TTC GAG TTA TGG GTA CA-3' for rolC (expected size of product 626 bp) according to Grabkowska et al. (2010) and 5'-ACT GAA TAT CAG GCA ACG CC-3' and 5'-GCG TCA AAG

AAA TAG CCA GC-3' for VirG (expected size of product 350 bp) according to Sidwa-Gorycka et al. (2009). The amplified fragments were separated in a 1.5% agarose gel and stained with ethidium bromide. The gels were documented with a Gel Doc<sup>TM</sup> XR + System (BioRad, München, Germany).

#### Hairy root submerged cultivation and growth analyses

*V. xanthophoeniceum* hairy root culture lines (VX1, VX2, VX5, VX6 and VX7) used in the experiments were grown in liquid MS medium in 250-mL Erlenmeyer flasks with 20% net volume shaken at 110 rpm, in the dark at 25°C. All flasks were inoculated with  $\sim 1.0 \text{ g}$  fresh 14-day-old roots per flask. The growth of the roots was assessed by harvesting cultures 2 and 3 weeks after inoculation. On each occasion, the hairy roots were separated from the culture medium by filtration. The final fresh and dried biomasses of the harvested roots were then gravimetrically determined (the latter after freeze-drying to constant weight). The growth of *V. xanthophoeniceum* hairy roots was monitored through determination of accumulated dry biomass (ADB) and growth index (GI) as described before (Georgiev et al. 2006). ADB = final dry biomass – initial dry biomass (g L<sup>-1</sup>); GI = (final dry biomass – initial dry biomass)/initial dry biomass.

#### Metabolite extraction and high performance liquid chromatography with diode array detection analysis

Freeze-dried *V. xanthophoeniceum* leaves and hairy roots samples ( $\sim 100 \text{ mg}$ ) were extracted with methanol (solid:liquid ratio 1:50) in an ultrasonic bath (3 × 20 min), pooled, filtered through 0.2-µm filters and directly injected into an high performance liquid chromatography with diode array detection (HPLC–DAD) system, to quantify the verbascoside content. The HPLC–DAD system consisted of an 1200 Series instrument equipped with a G1310A pump, a G1329A autosampler, a G1322A degasser, a G1316A column oven, a G1315D diode array detector controlled by ChemStation (all from Agilent Technologies, Inc., Santa Clara, CA, USA) and a Luna reverse phase (C18) column (150 × 4.6 mm i.d., 5 µm particle size; Phenomenex, Utrecht, The Netherlands). The metabolites were then separated using a mobile phase consisting of methanol/water 5:95 (A:B) for 3 min, followed by linear gradients to 25:75 (A:B) after 7 min then to 60:40 (A:B) after 13 min and the flow rate was 1 mL min<sup>-1</sup> (Gyurkovska et al. 2011). Eluting verbascoside was detected, and quantified, by monitoring the eluate at a wavelength of 330 nm. A standard calibration curve was plotted using various concentration ranges (0–400 µg mL<sup>-1</sup>) of verbascoside (a gift

from Dr. I. Koleva, University of Food Technology, Plovdiv, Bulgaria).

#### Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry analyses

Extracts from *V. xanthophoeniceum* leaves and hairy roots (10 µL) were injected into an Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) system consisting of an 1100 Series instrument equipped with a G1312A binary pump, a G1367A autosampler, a G1379A degasser, a G1316A column oven, a G1315B diode array detector, a simple quadrupole SL mass spectrometer driven by ChemStation software, all supplied by Agilent Technologies. The same column and mobile phase as described above were used. The eluent was monitored by the UV detector at 330 nm, and by the MS from mass-to-charge ratio ( $m/z$ ) 300–850 (negative ionization mode) with drying gas at 10 mL min<sup>-1</sup>, 350°C, 50 psig and capillary voltage at 3,000 V.

#### Statistical analysis of the data

All transformation experiments were repeated three times. The data from shake-flask cultures are averages obtained from three independent experiments with two biological replicates. HPLC–DAD quantitative measurements and LC-APCI-MS analyses were done in triplicate. The results are presented as mean ± standard errors (SE).

## Results and discussions

### Establishment of hairy root cultures

Three different strains of *A. rhizogenes*—ATCC 15834, LBA 9402 and TR 105—were tested for their ability to induce the formation of hairy roots on *V. xanthophoeniceum* explants. We performed a huge experimental scheme

with all *Agrobacterium* strains as both co-cultivation and direct infection methods were applied. Acetosyringone (in concentrations of 100 and 200 µM) was also added. Although over 600 explants were wounded and treated with *A. rhizogenes* (Table 1) no hairy root formation was observed. These data suggest that *V. xanthophoeniceum* plants are not susceptible to infection with *A. rhizogenes* possible due to the hairy leaf surface, which might be responsible for reduced access of Agrobacteria. Only one report exists in literature on the transformation of *V. phoeniceum* plants with *A. tumefaciens* (McCaskill and Turgeon 2007); however, there is no information available on the successful transformation of *Verbascum* plants with *A. rhizogenes* and hairy root induction (at least in SCOPUS database, accessed on 22.11.2010).

Thus we decided to apply the SAAT technology for *V. xanthophoeniceum* transformation. Based on the available literature data the exposure of wounded explants to ultrasound was chosen as 30, 45 and 60 s. The best performance was observed when explants were treated with ultrasound for 45 s, because in this case ~75% of explants treated with *A. rhizogenes* ATCC 15834 showed hairy roots formation (Table 1). In the other two conditions also hairy root growth was observed; however, the transformation frequency was significantly lower (15–28%) and even necrosis of mother plant tissue when exposed to 60 s ultrasound was observed (Table 1). Neoplastic hairy roots growth at the wound site of *Verbascum* leaves occurred already after 7 days of culture (10 days after transformation), which significantly reduces the time for hairy roots induction (Fig. 1). Furthermore, we successfully transformed via the above-described SAAT protocol *V. densiflorum* (using *A. rhizogenes* ATCC 15834) and *V. phlomoides* (with both *A. rhizogenes* strains ATCC 15834 and LBA 9402), which proves the high reliability of the SAAT technology for the transformation of *Verbascum* species. The infection frequency of ATCC 15834 strain was similar to that reported above (77 and 72% in the case of *V. densiflorum* and *V. phlomoides*, respectively), while

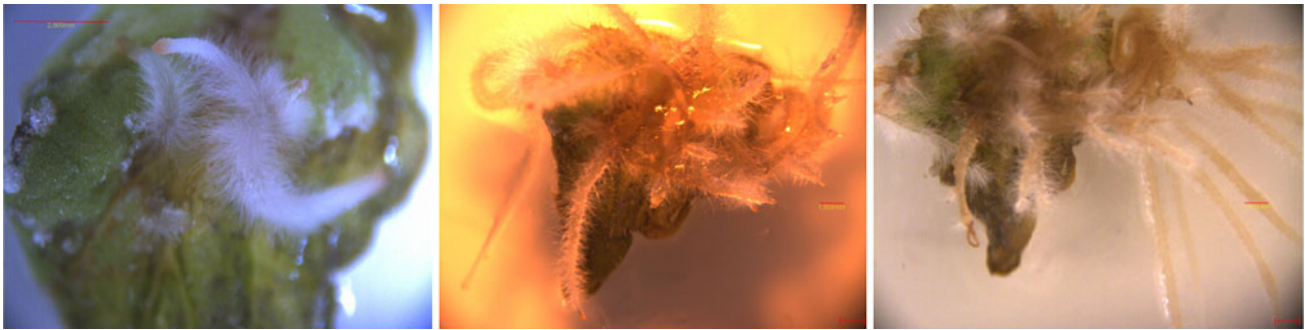
**Table 1** Frequency of hairy root formation of *Verbascum xanthophoeniceum*

<i>Agrobacterium</i> strain	Method for transformation	Number of explants	Infection frequency (%)	Total number of hairy roots after 21 days
ATCC 15834	Co-cultivation/direct infection <sup>a</sup>	100/115	0	0
LBA 9402	Co-cultivation/direct infection <sup>a</sup>	105/105	0	0
TR 105	Co-cultivation/direct infection <sup>a</sup>	100/105	0	0
ATCC 15834	SAAT (30 s) <sup>b</sup>	54	60	227
	SAAT (45 s)	65	75	452
	SAAT (60 s)	52	47	164

<sup>a</sup> To half of the explants acetosyringone (100 or 200 µM) was added

<sup>b</sup> Exposure of explants to ultrasound is given in parenthesis



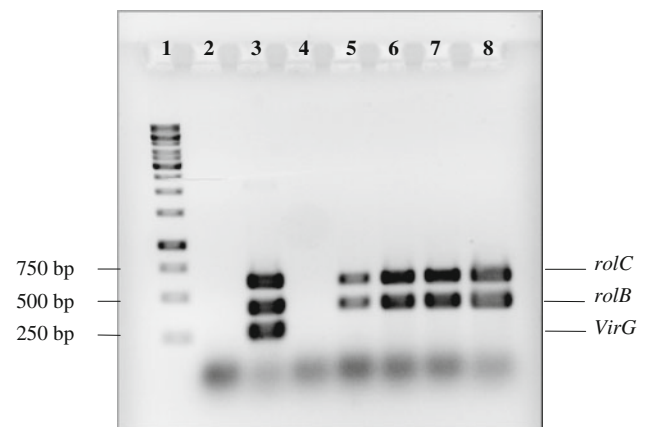


**Fig. 1** Hairy roots formation via sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum*. From left to right—10, 17 and 21 days after transformation

when LBA 9402 was used for transformation of *V. phlomoides* the infection frequency was over 90% (data not shown). In all cases first neoplastic roots appeared 10–14 days after transformation. However, it should be noted that the time for hairy roots formation depends on species, age and type of plant tissue. For instance, when *Papaver* hypocotyls were infected with *A. rhizogenes* LBA 9402 via ultrasound, hairy roots occurred after 5 weeks of culture (Le Flem-Bonhomme et al. 2004).

After 3 weeks of cultivation 10 hairy root lines were isolated from different explants, denoted as VX1–10. When excised and transferred to new MS solid media three hairy root lines showed bacterial contamination (lines VX3, VX4 and VX10), which was not possible to be eliminated even after repeated antibiotic treatment. Two other lines (VX8 and VX9) did not show any growth when hairy roots were excised from mother explants and transferred to a new media. All remaining hairy roots lines (VX1, VX2, VX5, VX6 and VX7) showed stable growth and absence of bacterial contamination. *V. xanthophoeniceum* VX1 and VX6 lines showed typical morphology of thick roots with high degree of lateral branching, plagiotropic root growth and profusion of root hairs, while VX2, VX5 and VX7 lines differed in morphology, grew like fine thin branched roots.

To confirm the complete and stable genetic transformation, PCR analyses of DNA from two *V. xanthophoeniceum* lines VX1 and VX6 with primer pairs for the amplification of *rolB*, *rolC*, and *VirG* (Fig. 2) were performed. Although the molecular mechanism of hairy roots induction is not completely understood, it is known that the agropine type of *A. rhizogenes* transfers two independent T-DNAs, denoted T<sub>L</sub>-DNA and T<sub>R</sub>-DNA, to the host plant genome (Nilsson and Olsson 1997). Both fragments are independently transferred and integrated to the host genome; however, the transfer of T<sub>L</sub>-DNA is essential to induce hairy roots. The sequence analyses of T<sub>L</sub>-DNA identified 18 open reading frames (ORF), four of which are essential for hairy root induction, namely ORF10 (*rolA*),



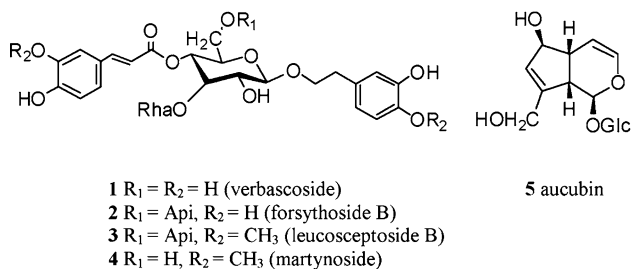
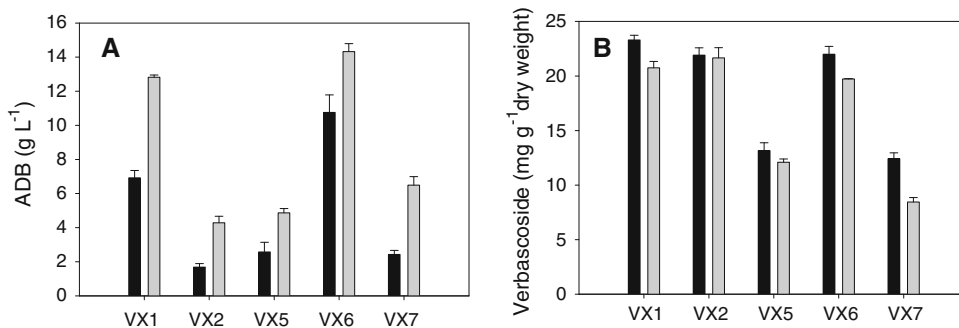
**Fig. 2** PCR analysis of DNA from transformed *Verbascum xanthophoeniceum* root lines VX1 and VX6: lane 1 contains GeneRuler™ (Fermentas); lanes 2–8 contain amplified PCR products with different templates: 2 DNA from untransformed *Verbascum* roots (negative control); 3 Ri plasmid which is *rolC* (626 bp), *rolB* (423 bp), and *VirG* (350 bp) positive; 4 water control; 5, 6 DNA from VX1 (first and second isolation); 7, 8 DNA from VX6 (first and second isolation)

ORF11 (*rolB*), ORF12 (*rolC*) and ORF15 (*rolD*; Nilsson and Olsson 1997). Amplification of *rolB* and *rolC* primers showed 423 and 626 bp bands for the transformed root, respectively (Fig. 2, lanes 5–8), which is a proof for the successful genetic transformation of lines VX-1 and VX-6. No band for the *VirG* genes in untransformed *Verbascum* roots (lane 4) and transformed root culture lines (lanes 5–8) was observed, which is an indication for the absence of bacterial contamination in the roots. When the Ri plasmid of *A. rhizogenes* was analyzed it gave three PCR fragments as expected (lane 3). As negative control, untransformed *Verbascum* roots were also analyzed to demonstrate the specificity of the primer pairs (Fig. 2, lane 2).

#### Hairy roots submerged cultivation and chemical analyses

*V. xanthophoeniceum* hairy roots lines VX1, VX2, VX5, VX6 and VX7 were grown 4 months on solid MS medium

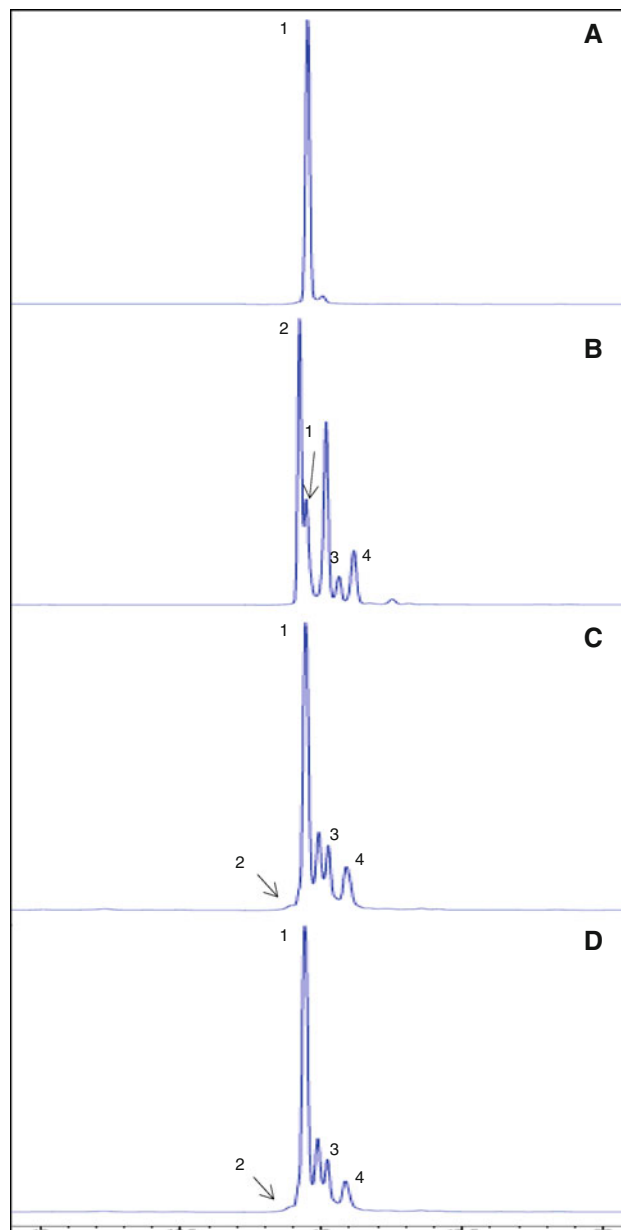
**Fig. 3** Time courses of growth (a) and verbascoside accumulation (b) in *Verbascum xanthophoeniceum* hairy roots, cultivated in shake-flasks. Black bars 14 days of culture, gray bars 21 days of culture. Mean values  $\pm$  SE



**Fig. 4** Chemical structures of phenylethanoid and iridoid glycosides isolated from *Verbascum xanthophoeniceum* hairy root culture lines VX1 and VX6

with 21-day period of subculture. That period was enough to get hairy roots with stable growth and morphological characteristics and for multiplication of hairy roots mass. Furthermore, we performed submerged cultivation of all five hairy root lines and the root mass was harvested after 2 and 3 weeks of culture (Fig. 3a). The results indicate that the most significant growth with a large number of lateral branching was observed for hairy root lines VX6 and VX1, which accumulated  $14.33 \pm 0.47$  and  $12.82 \pm 0.13$  g L<sup>-1</sup> of biomass, respectively. Lines VX2, VX5 and VX7, although showing visible multiplication of root mass, accumulated lower biomass amounts ( $<7$  g L<sup>-1</sup>; Fig. 3a). Calculated GI values 3 weeks after inoculation were 6.9–7.8 for best growing VX6 and VX1 lines. These values are similar or slightly higher than those reported for hairy root cultures of *Beta vulgaris* and *Harpagophytum procumbens* (Pavlov et al. 2007; Homova et al. 2010).

HPLC–DAD analyses of *V. xanthophoeniceum* hairy roots lines revealed the presence of significant amounts of verbascoside (Fig. 3b). The highest content of verbascoside was detected in VX1, VX2 and VX6 lines ( $23.31 \pm 0.42$ ,  $21.90 \pm 0.66$  and  $22.00 \pm 0.72$  mg g<sup>-1</sup>, respectively). It is worth mentioning that these amounts were over six times higher compared to the verbascoside content in the original *V. xanthophoeniceum* leaves used for transformation ( $3.48 \pm 0.02$  mg g<sup>-1</sup> dry weight). Verbascoside levels in transformed root cultures (2.19–2.33%) are also several times higher compared to those detected in the flowers of *V. densiflorum* (0.688–0.742%) and *V. phlomoides*



**Fig. 5** Representative HPLC chromatograms of pure verbascoside **a** *V. xanthophoeniceum* leaves **b** and hairy root lines VX1 **c** and VX6 **d** Peaks 1 verbascoside, 2 forsythoside B, 3 leucosceptoside B, 4, martynoside

(0.167–0.206%; Klimek et al. 2010). Verbascoside (also known as acteoside, kusagin and orobanchin; Fig. 4) is a very active member of the phenylethanoid group, which has been isolated from many plant species (Dembitsky 2005). It exhibits a wide spectrum of biological activities, including anti-leukemic and cytotoxic activity against a murine cell line (Pettit et al. 1990) and anti-inflammatory activity through inhibition of cyclooxygenase-2 expression and inhibition of complement activity in human serum (Gyurkovska et al. 2011). Verbascoside was reported as major compound in hairy root cultures of *Gmelina arborea* and *H. procumbens*, however, in significantly lower amounts than in *V. xanthophoeneum* (0.91 and 1.1 mg g<sup>-1</sup> dry weight, respectively; Dhakulkar et al. 2005; Homova et al. 2010). Therefore, *V. xanthophoeneum* VX1 and VX6 hairy root clones might be attractive producers of bioactive verbascoside.

In addition, we partially analyzed the metabolite profiles of methanolic extracts of *V. xanthophoeneum* hairy roots by LC-APCI-MS. Except verbascoside with *m/z* 623.2 [M]<sup>-</sup> (1) two other phenylethanoid glycosides, leucosceptoside B with *m/z* 783.2 [M]<sup>-</sup> (3) and martynoside with *m/z* 651.2 [M]<sup>-</sup> (4) and one iridoid glycoside, aucubin with *m/z* 345.2 [M]<sup>-</sup> (5), were identified in both mother plant leaves and *V. xanthophoeneum* hairy root lines VX1 and VX6. Typical HPLC–DAD chromatograms are presented in Fig. 5. Forsythoside B (*m/z* 755.2 [M]<sup>-</sup>; (2) was detected only in small amounts in transformed root cultures in opposite of intact *Verbascum* leaves, where it appeared as the most abundant phenylethanoid glycoside (Fig. 5B). On the contrary, the levels of verbascoside were higher in the hairy roots (Fig. 5C, D) than in intact leaves, indicating a possible shift in the biosynthetic pathways. It should be noted that aucubin could not be seen at these conditions (330 nm) as it shows absorbance maximum at 204 nm.

## Conclusion

*V. xanthophoeneum* plant leaves were not susceptible to transformation with three *A. rhizogenes* strains when co-cultivation and direct infection methods (with or without addition of acetosyringone) were applied. Therefore, we developed an efficient SAAT protocol for *Verbascum* hairy root culture induction. Most vigorous hairy root clones showed stable growth under submerged cultivation and accumulated high biomass amounts. Verbascoside was the most abundant secondary metabolite and its amounts in hairy root clones were several times higher than in mother plant tissue. Therefore, these hairy root cultures will be promising material for possible large scale production of this bioactive metabolite in the future.

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