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ORIGINAL RESEARCH PAPER

Genetic transformation of rare *Verbascum eriophorum* Godr. plants and metabolic alterations revealed by NMR-based metabolomics

Andrey Marchev · Zhenya Yordanova · Kalina Alipieva · Georgi Zahmanov · Snezhana Rusinova-Videva · Veneta Kapchina-Toteva · Svetlana Simova · Milena Popova · Milen I. Georgiev

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Abstract
Objectives To develop a protocol to transform *Verbascum eriophorum* and to study the metabolic differences between mother plants and hairy root culture by applying NMR and processing the datasets with chemometric tools.
Results *Verbascum eriophorum* is a rare species with restricted distribution, which is poorly studied. *Agrobacterium rhizogenes*-mediated genetic transformation of *V. eriophorum* and hairy root culture induction are reported for the first time. To determine metabolic alterations, *V. eriophorum* mother plants and relevant hairy root culture were subjected to comprehensive metabolomic analyses, using NMR (1D and 2D). Metabolomics data, processed using chemometric tools (and principal component analysis in particular) allowed exploration of *V. eriophorum* metabolome and have enabled identification of verbascoside (by means of 2D-TOCSY NMR) as the most abundant compound in hairy root culture.
Conclusion Metabolomics data contribute to the elucidation of metabolic alterations after T-DNA transfer to the host *V. eriophorum* genome and the development of hairy root culture for sustainable bioproduction of high value verbascoside.

Keywords *Agrobacterium rhizogenes* · Hairy roots · Iridoids · Mullein · Phenylethanoids · Verbascoside · *Verbascum eriophorum*

Introduction

The genus *Verbascum* L. (mulleins, Scrophulariaceae) comprises more than 360 species of plants widely distributed all over the globe. Different parts of the plants have a longstanding use in traditional medicine and have been applied as anti-inflammatory remedies in the treatment of eczema and in wound healing and in respiratory tract disorders (Alipieva et al. 2014b). Among other bioactive molecules that accumulate in *Verbascum* spp., iridoid and phenylethanoid glycosides are the major and most important ones (Georgiev et al. 2011, 2012, 2013; Alipieva et al. 2014b). Phenylethanoid glycosides are water-soluble compounds widely distributed in the plant kingdom,

A. Marchev · G. Zahmanov · S. Rusinova-Videva · M. I. Georgiev (✉)
Laboratory of Applied Biotechnologies, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 139 Ruski Blvd., Plovdiv 4000, Bulgaria
e-mail: milengeorgiev@gbg.bg

Z. Yordanova · V. Kapchina-Toteva
Department of Plant Physiology, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd., Sofia 1164, Bulgaria

K. Alipieva · S. Simova · M. Popova
Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

characterized by cinnamic acid (C_6–C_3) and hydroxyphenylethyl (C_6–C_2) moieties attached to a β-glucopyranose (apiose, galactose, rhamnose, xylose, etc.) via a glycosidic bond (Alipieva et al. 2014a). A second group of natural compounds are iridoids with a six-membered ring containing an oxygen atom fused to a cyclopentane ring (Geu-Flores et al. 2012). Both groups have gained interest as active ingredients in treatment of various health disorders.

Global over-harvesting of several valuable plant species, along with the significant reduction of plant biodiversity, is forcing the development of innovative technologies, that are likely to bring plant cell/tissue culture technology to the forefront (Georgiev and Weber 2014). Among other bioproduction platforms, transformed root culture (= hairy roots)-induced via *Agrobacterium rhizogenes*-mediated genetic transformation—seems to be one of the most interesting and efficient for mass production of plant-derived metabolites for various purposes (Ludwig-Müller et al. 2014). Moreover, hairy roots offer an immense potential for biosynthetic pathways elucidation and to engineer the biosynthesis of targeted molecules (Ludwig-Müller et al. 2014; Runguphan et al. 2010).

Metabolomics, as a part of the systems biology, explores the chemical fingerprints (in both qualitative and quantitative fashion) that metabolic processes in living organisms at certain conditions leave behind (Halabalaki et al. 2014; Kim et al. 2011; Nicholson 2010). Comprehensive metabolic profiling and metabolomics datasets are important to identify the spatial and temporal distribution of target specialized metabolites and serves as constrain towards reconstruction of metabolic models, hence metabolomics takes central role in functional genomics (Rai and Saito 2016; Tohge and Fernie 2010). NMR is widely used for the simultaneous detection of single compounds in highly complex mixtures along with their quantification as the signal intensity (integral) is directly proportional to the number of protons (Kim et al. 2010, 2011; Simmler et al. 2014). In our research program we have set-up and applied an NMR-based metabolomics platform in tandem with multivariate data analysis to study metabolic differentiations of the genera *Verbascum* (Georgiev et al. 2011) and *Sambucus* (Zahmanov et al. 2015) as well as compare *V. nigrum* mother plant tissue and SAArT-mediated (sonication assisted *A. rhizogenes*-mediated transformation) hairy roots (Georgiev et al. 2015). Saiman et al. (2015) have also used NMR-based metabolomics to portray secondary metabolites and location of five-carbon precursors in jasmonic acid-elicited *Catharanthus roseus* in vitro systems, hence widening the range of its application.

Verbascum eriophorum Godr. (The Plant List) is a Balkan endemic plant under the protection of the Bulgarian biodiversity law with national conservation status as "rare", hence included in the Red Data Book of Bulgaria (Velchev et al. 1984). The species is distributed in the Balkan Peninsula, e.g. northern Greece, Bulgaria and the former Yugoslavia. *V. eriophorum* is a very tall (3 m ht) biennial herbaceous plant with grey basal leaves, dense and woolly flower stalks and could be confused with *V. phlomoides*, which is, however, shorter (www.gardensnorth.com; accessed on 07.03.2016). The aim of our study was to develop a protocol for *V. eriophorum* Godr. transformation as well as to study the metabolic differences between mother plants and hairy root culture by applying 1D- and 2D-NMR and further processing the datasets with chemometric tools. As far as we know this is the first report on the genetic transformation of rare *V. eriophorum* plants along with the application of metabolomics approach for a holistic look on metabolome changes as a result of the foreign T-DNA transfer.

Materials and methods

Intact plant material and *V. eriophorum* in vitro shoot culture induction

Verbascum eriophorum seeds were obtained with courtesy of Medicinal Plant Garden, Department of Pharmacognosy with Medicinal Plant Unit (Medical University of Lublin, Poland). The origin of plant material is from Göteborg Botanical Garden, Göteborg, Sweden (accession number 123). Initiation of in vitro shoot culture was accomplished by surface sterilization of *V. eriophorum* seeds with 70 % (v/v) ethanol for 5 min, followed by washing with 96 % (v/v) ethanol for 10 s. Under aseptic conditions, seeds were inoculated on half-strength MS medium (Murashige and Skoog 1962), solidified with 0.7 % (w/v) agar. After two weeks, seeds were transferred on basal MS medium, supplemented with 3 % (w/v) sucrose and agar as described above. Seedlings were grown

under controlled environmental conditions [with 16 h of illumination period, 60 μmol $(m^2\ s)^{-1}$ photosynthetic photon flux density, Philips TLD-33, at 25 °C with 60–70 % air humidity] for 30 days, followed by a transfer to a fresh MS media, supplemented with different concentrations of N^6-benzyladenine (0.1, 0.5, and 1 mg BA l^{-1}). Micropropagated plants were transferred on hormone-free basal MS medium and cultivated twice under controlled conditions (as above) for 45 days each before performing *A. rhizogenes* infection.

Genetic transformation with *A. rhizogenes*

Verbascum eriophorum in vitro leaves (third and fourth stage fully developed leaves) were infected with *A. rhizogenes* ATCC 15834 (see Georgiev et al. 2015). Prior to experiments, *A. rhizogenes* was suspended in MS media to an OD_{600} of 0.8. Both direct infection method and SAArT protocol (Georgiev et al. 2015) were used for genetic transformation of aseptic *V. eriophorum* explants (ca. 1 cm^2).

Genomic DNA isolation and PCR amplification

Total genomic DNA from three hairy root clones of *V. eriophorum* (denoted as VE-D1, VE-D2, VE-D3) was extracted using a DNeasy Plant Mini Kit (Qiagene, Germany). Plasmid DNA (pRi) from *A. rhizogenes* was isolated using QIAprep Spin Miniprep Kit (Qiagene). A primer pair of 5′-GCT CTT GCA GTG CTA GAT TT-3′ and 5′-GAA GGT GCA AGC TAC CTC TC-3′ was used to amplify a 423 bp fragment of *rolB* gene and a second pair of primers, 5′-CTC CTG ACA TCA AAC TCG TC-3′ and 5′-TGC TTC GAG TTA TGG GTA CA-3′ was used to amplify a 626 bp fragment of *rolC* gene. In addition, primers 5′-ACT GAA TAT CAG GCA ACG CC-3′ and 5′-GCG TCA AAG AAA TAG CCA GC-3′ amplifying a fragment of 350 bp were used for detecting the *virG* gene. The reactions were performed with Multiplex PCR Plus Kit (Qiagene) containing 20 ng plant genomic DNA (or 10 ng pRi DNA) and 0.2 μM of each primer. Conditions for *rolB*, *rolC*, and *virG* amplification were as follow: initial denaturation at 95 °C for 5 min, 35 cycles of amplification (95 °C 30 s, 57 °C 90 s, and 72 °C 1 min) and 10 min final extension at 68 °C. PCR amplicons were visualized after electrophoresis in 1.5 % (w/v) agarose gels.

Cultivation of hairy roots from *V. eriophorum*

Verbascum eriophorum hairy root clones (VE-D1, VE-D2, VE-D3) were obtained through cutting the root tips (ca. 1 cm long) from the mother plant leaf tissue and transferring them to a solid MS medium. Further, hairy root clones of *V. eriophorum* (VE-D1 and VE-D2) were grown in 250 ml Erlenmeyer flasks (20 % net volume of liquid MS medium), shaken at 110 rpm, in the dark at 26 °C. Each Erlenmeyer flask was inoculated with ca. 1.5 g fresh weight of two weeks old roots. Accumulated dry biomass (ADB) and growth index (based on dry weight; GIDW) were determined aiming to monitor the growth of the hairy roots. ADB = Final dry biomass − initial dry biomass (g l^{-1}); GIDW = (Final dry biomass − initial dry biomass)/initial dry biomass.

Metabolomics extraction protocol and NMR conditions

50 mg freeze-dried ground leaves of *V. eriophorum* (five biological replicates) as well as hairy roots (four biological replicates) were homogenized with equal amounts of CD_3OD (0.75 ml) and D_2O (KH_2PO_4 buffer, pH 6), containing 0.005 % (w/v) TSPA-d_4 (0.75 ml). After 20 min of ultrasonication (35 kHz; UCI-50Raypa R. Espinar S.L., Barcelona, Spain) samples were centrifuged (14,000×*g*, 20 min) and the supernatant was analysed by NMR as described by Kim et al. (2010).

Proton (^{1}H) as well as 2D-NMR spectra (*J*-resolved, COSY, HSQC and TOCSY) were recorded at 25 °C on a AVII + 600 spectrometer (Bruker, Karlsruhe, Germany), operating at a proton NMR frequency of 600.11 MHz (Georgiev et al. 2015). For internal lock deuterated methanol was used and the relaxation time accounts on 4.07 s. The resulting ^{1}H-NMR spectra were further processed by referencing to the internal standard trimethylsilyl propanoic acid (TSPA), phased and baseline corrected, by running MestReNova software (v10.0, Mestrelab Research, Santiago de Compostela, Spain). CD_3OD and D_2O from Deutero GmbH (Kastellaun, Germany) were used in experiments.

NMR data analyses

The ^{1}H-NMR and the 2D data files were further processed as described before by Zahmanov et al.

(2015). In brief, all spectra were normalized; spectral intensities were scaled to TSPA and bucketed to 0.04 ppm integrated regions of equal width. The parts of NMR spectra corresponding to the residual signals of water (δ 4.80–4.84) and methanol (δ 3.30–3.34) were automatically excluded from the chemometrics analyses. SIMCA-P software package (v12.0, Umetrics, Umeå, Sweden) was used to perform principal component analysis with Pareto scaling method.

Results and discussion

Induction of hairy root culture and growth analysis

The high importance of *Verbascum*-derived bioactive molecules along with the limited access to *V. eriophorum* are the driving forces for the development of an alternative to classical approaches for plant growth and sustainable supply of high value molecules.

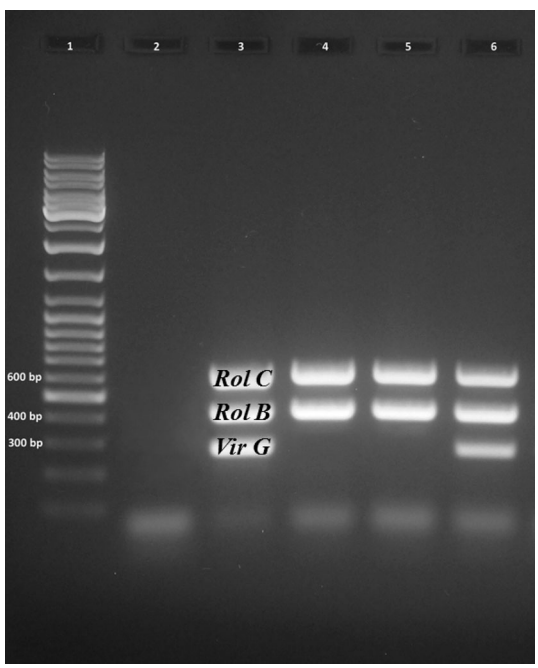

Fig. 1 PCR analysis of genomic DNA from *V. eriophorum* Godr. and hairy root culture clones (VE-D1, VE-D2 and VE-D3). *Lane 1* GeneRuler (Thermo Scientific); *2* negative control (*V. eriophorum* DNA); *3* positive control (Ri plasmid, containing *rol C*, *rol B*, and *Vir G*); *4*, *5* and *6* DNA from hairy roots VE-D1, VE-D2 and VE-D3, respectively

The micropropagation is an essential method for ex situ conservation of rare and threatened plants, species with reduced populations and low fertility, and for fast propagation of valuable medicinal plants, as well. The in vitro multiplication of *V. eriophorum* was achieved on MS medium supplemented with BA (0.1, 0.5, and 1 mg l^{-1}); BA at 0.5 mg l^{-1} was the most effective in promoting shoot development and approx. 98 % of explants showed shoot proliferation and produced ~14 shoots per explant. BA suppressed root development and stimulated callus formation. The effective response of BA in stimulating shoot formation was also reported for *V. thapsus* by Turker et al. (2001). Successful rooting of micropropagated plants was achieved on hormone-free basal MS medium and 67 % formed a well-developed root system and plentiful leaf biomass. Before performing the transformation analyses in vitro regenerated plants were cultivated twice on basal MS medium under controlled environmental conditions (as described above) for 45 days each. An efficient protocol for genetic transformation of *V. nigrum* L. with *A. rhizogenes* was developed early by utilizing SAArT (Georgiev et al. 2015) and, like previously, 45 s sonication treatments for the transformation of *V. eriophorum* plants along

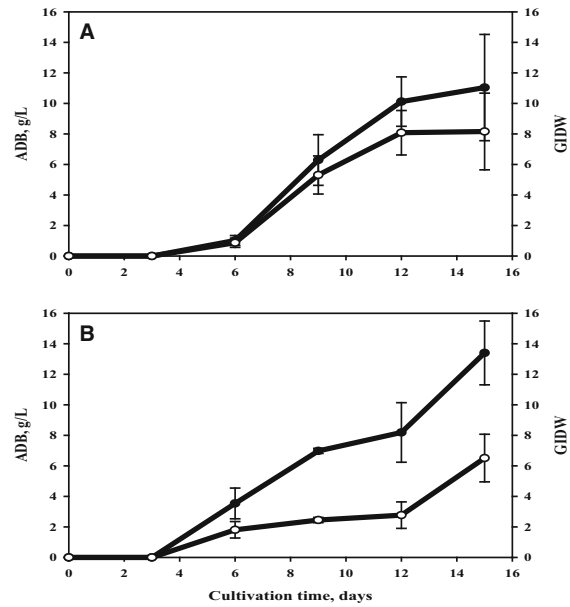

Fig. 2 Time course of growth of *V. eriophorum* Godr. hairy root culture clones VE-D1 **(a)** and VE-D2 **(b)** in shaken-flasks. *ADB* accumulated dry biomass (*filled circle*), *GIDW* growth index dry weight (*open circle*)

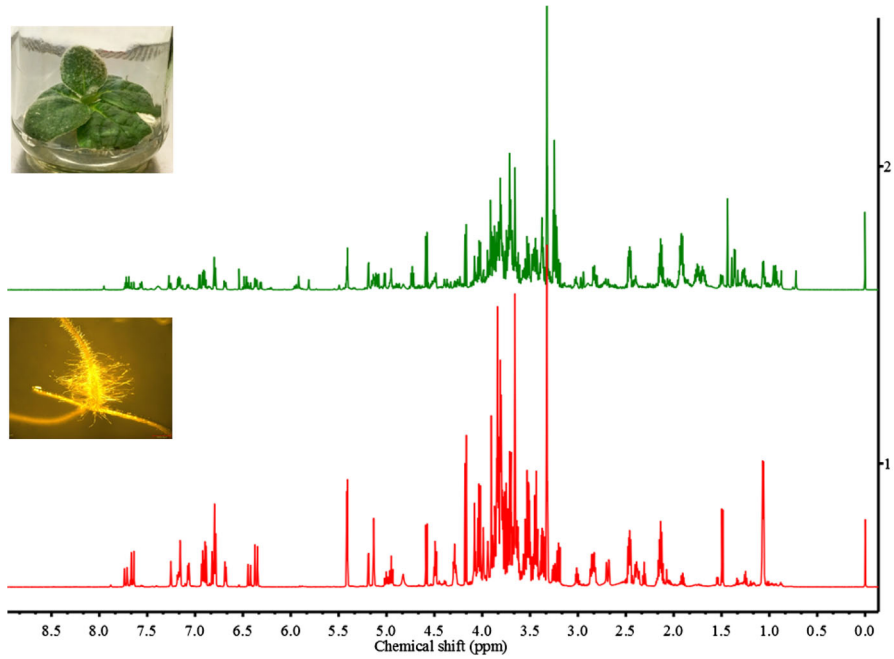

Fig. 3 Proton NMR spectra of *V. eriophorum* Godr. intact plants (*top*) and hairy root culture (*bottom*)

with direct infection method were subsequently applied. Both transformation approaches resulted in hairy roots induction with infection frequency of ca. 50 %; however only hairy root clones, generated by applying direct infection method were able to grow further on MS solid media.

The three best growing hairy root clones (VE-D1, VE-D2 and VE-D3), showing typical hairy root phenotype with excessive branching and growth in hormone-free medium, were further selected for PCR analyses. To confirm successful genetic transformation primers for the amplification of *rolB*, *rolC* and *virG* genes were used (Fig. 1). As an agropine type the *A. rhizogenes* strain ATCC 15834 transfers to the *V. eriophorum* genome two independent T-DNAs, denoted as T_L-DNA and T_R-DNA. T_L-DNA harbors ORFs 10 (*rolA*), 11 (*rolB*), 12 (*rolC*) and 15 (*rolD*), which appear essential for induction of hairy root culture, hence their amplification would be an enough adequate proof for the successful T-DNA transfer (Georgiev et al. 2015). PCR results indicate that all three hairy root clones—VE-D1, VE-D2 and VE-D3—give bands at ca. 620 bp and 420 bp for *rolC* and *rolB*, respectively (Fig. 1, lanes 4–6). The genomic DNA from non-hairy *V. eriophorum* plants did not show any bands for *rol* genes (Fig. 1, lane 2). Further, we also examined the amplification of *virG* gene (350 bp) in *V. eriophorum* hairy roots as an indicator for presence of bacterial contamination in hairy root culture. Surprisingly, *virG* amplification in VE-D3 clone indicated contamination (Fig. 1, line 6), although all hairy root clones were grown for 21 days on MS media, supplemented with antibiotic (cefotaxime) for bacteria elimination. Under certain conditions, *A. rhizogenes* could be present in hairy root culture without exhibiting significant growth, detectable under visual observation; hence amplification of *vir* genes products is mandatory requirement in order to prove them as free of bacterial contamination.

We performed submerged cultivation of VE-D1 and VE-D2 hairy root clones in shake-flasks (Fig. 2a, b). The results indicate stable growth under submerged conditions with profound lateral branching, as at the end of the cultivation period accumulated dry biomass of VE-D1 and VE-D2 peaked at 11 and 13.4 g l^{-1}, respectively. Calculated GIDW values 15 days after inoculation were 6.5–8.2. Hairy root clone VE-D2 was further selected for NMR-based metabolomics analyses.

Metabolomic studies of *V. eriophorum*

In order to reveal metabolic alterations as a result of foreign gene transfer from *Agrobacterium* to the *V.*

Table 1 *Verbascum eriophorum* Godr. metabolites, identified in intact plants and hairy root culture by relevant 1D and 2D NMR spectra

Metabolite	Mother plant leaves[a]	Hairy root culture[a]	Selected signals, multiplicity and coupling constant[b]
Alanine	+	+++	δ 1.49 (d, $J = 7.2$)
Glutamine	+	++	δ 2.13 (m)/δ 2.46 (m)/δ 3.72 (t)
Threonine	+	–	1.36 (d, $J = 6.3$)
Valine	+	+	δ 1.01 (d, $J = 7.0$)/δ 1.06 (d, $J = 7.0$)
α-Glucose	+	+	δ 5.19 (d, $J = 3.8$)
β-Glucose	+	+	δ 4.58 (d, $J = 7.9$)
Sucrose	+	+++	δ 5.41 (d, $J = 3.8$)/δ 4.17 (d, $J = 8.7$)
Acetic acid	++	–	δ 1.95 (s)
Succinic acid	+	–	δ 2.51 (s)
Formic acid	–	+	δ 8.48 (s)
Fumaric acid	++++	+	δ 6.53 (s)
Malic acid	++	+	δ 2.81 (dd, $J = 16.9, 8.3$)/δ 2.95 (dd, J = 16.9, 3.9)
Choline	+	+	δ 3.21 (s)
Harpagide	+	–	δ 6.07 (d, $J = 0.98$)
Aucubin	+	–	δ 6.32 (dd, $J = 6.2, 1.9$)/δ 5.81 (brs)/δ 5.11 (dd, $J = 6.2, 3.7$)/δ 5.09 (d, $J = 6.1$)/δ 4.34 (d, $J = 15.6$)/δ 4.22 (d, $J = 15.2$)
p-Coumaroyl rhamnopyranosyl aucubin	+	–	δ 7.72 (d, $J = 15.9$)/δ 7.57 (d, $J = 8.8$)/δ 7.56 (d, $J = 8.8$)/δ 6.91 (d, $J = 8.4$)/δ 6.43 (d, $J = 15.9$)/δ 6.37 (dd, $J = 6.2, 1.8$)/δ 5.92 (brs)/δ 5.15 (dd, $J = 6.2, 3.8$)/δ 5.02 (d, $J = 6.6$)/δ 4.74 (d, $J = 7.8$)
Feruloyl rhamnopyranosyl aucubin	+	–	δ 7.71 (d, $J = 15.9$)/δ 7.28 (d, $J = 1.9$)/δ 7.18 (dd, $J = 8.3, 1.9$)/δ 6.91 (d, $J = 8.4$)/δ 6.47 (d, $J = 15.9$)/δ 6.37 (dd, $J = 6.2, 1.8$), δ 5.92 (brs)/δ 5.15 (dd, $J = 6.2, 3.8$)/δ 5.02 (d, $J = 6.6$)/δ 4.73 (d, $J = 7.8$)
Verbascoside	+	++++	δ 1.06 (d, $J = 6.2$)/δ 2.84 (m)/δ 4.49 (d, $J = 7.9$)/δ 5.13 (d, $J = 1.6$)/δ 6.36 (d, $J = 15.9$)/δ 6.87 (dd, $J = 8.1, 2.0$)/δ 6.79 (d, $J = 8.1$)/δ 6.80 (d, $J = 2.0$)/δ 6.89 (d, $J = 8.3$)/δ 7.07 (dd, $J = 8.3, 2.0$)/δ 7.16 (d, $J = 2.0$)/δ 7.66 (d, $J = 15.9$)
Martynoside	+	+++	δ 1.07 (d, $J = 6.1$)/δ 2.84 (m)/δ 4.50 (d, $J = 7.3$)/δ 5.13 (d, $J = 1.6$)/δ 6.44 (d, $J = 15.9$)/δ 6.80 (dd, $J = 8.2, 1.7$)/δ 6.83 (d, $J = 2.0$)/δ 6.92 (d, $J = 8.0$)/δ 6.93 (d, $J = 8.0$)/δ 7.18 (dd, $J = 8.3, 1.9$)/δ 7.27 (d, $J = 1.9$)/δ 7.73 (d, $J = 15.9$)

[a] The number of "+" refers to relative fold differences and "−"to absence of the particular compound

[b] Proton NMR chemical shifts (δ) and coupling constant (J)

(Georgiev et al. 2011, 2015; Saiman et al. 2015; Zahmanov et al. 2015)

eriophorum host plant genome, ^{1}H and 2D NMR analyses (*J*-resolved, COSY, HSQC and TOCSY) were performed, following previously described conditions (Zahmanov et al. 2015). Significant differences, both qualitative and quantitative, were noted between the *V. eriophorum* plants (grown in vitro) and hairy roots clone VE-D2 (Fig. 3). Among diversity of primary and secondary metabolites in *V. eriophorum* samples, amino and organic acids, carbohydrates, phenolic molecules and iridoid glycosides were identified (Table 1). Glutamine accumulated in higher amounts in hairy root culture than in the mother leaf tissue. This has also been noted with *V. nigrum* L. hairy roots (Georgiev et al. 2015). Signals of iridoid glycosides (harpagoside, aucubin and aucubin derivatives as *p*-coumaroyl rhamnopyranosyl aucubin and feruloyl rhamnopyranosyl aucubin) were characteristic for mother plant leaves only as well as threonine,

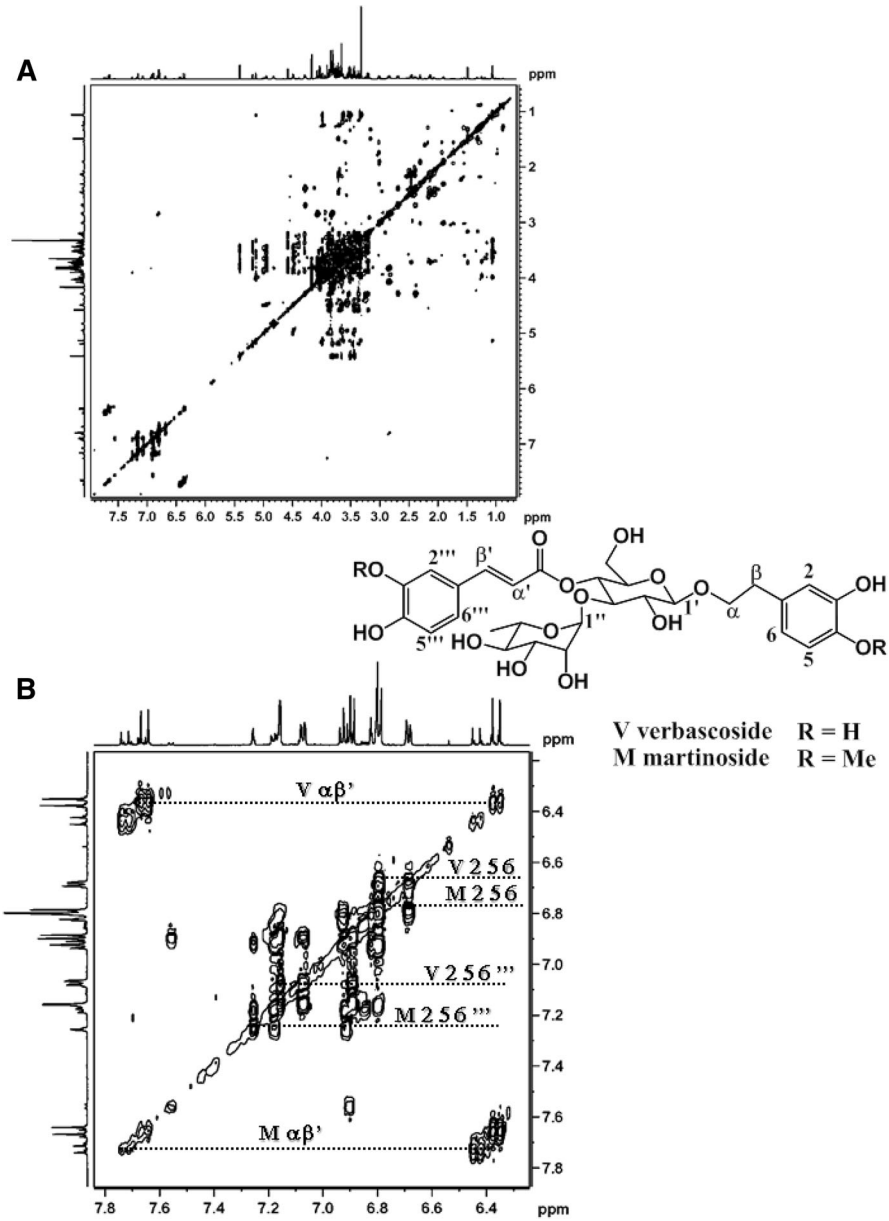

Fig. 4 Total correlation spectroscopy (^{1}H–^{1}H TOCSY) NMR spectra of hairy root culture extract of *V. eriophorum* Godr. **(a)** and phenylethanoid part of the spectrum with chemical structures of verbascoside and martynoside **(b)**

acetic and succinic acids, while hairy root culture was abundant in phenylethanoid glycosides (Table 1).

To elucidate the structures of the phenylethanoid glycosides, occurring in highest amount in *V. eriophorum* hairy roots (Table 1; Fig. 3) we used 2D NMR. By means of ^{1}H–^{1}H total correlation spectroscopy (TOCSY) spectra the structures were identified as verbascoside and martynoside (Fig. 4). Verbascoside (= acteoside) appeared the most abundant metabolite from phenylethanoids pool and its amounts in *V. eriophorum* VE-D2 clone were higher than in the mother leaf (Table 1; Fig. 3). Verbascoside has a valuable pharmacological profile, exhibiting anti-inflammatory, antineoplastic and cytoprotective (strong activator of NRF2—nuclear factor—erythroid 2 p45-related factor 2) activities, besides abundant wound-healing and neuroprotective properties (Alipieva et al. 2014a). It is therefore highly relevant to develop sustainable approaches for mass production of verbascoside for pharmaceutical applications.

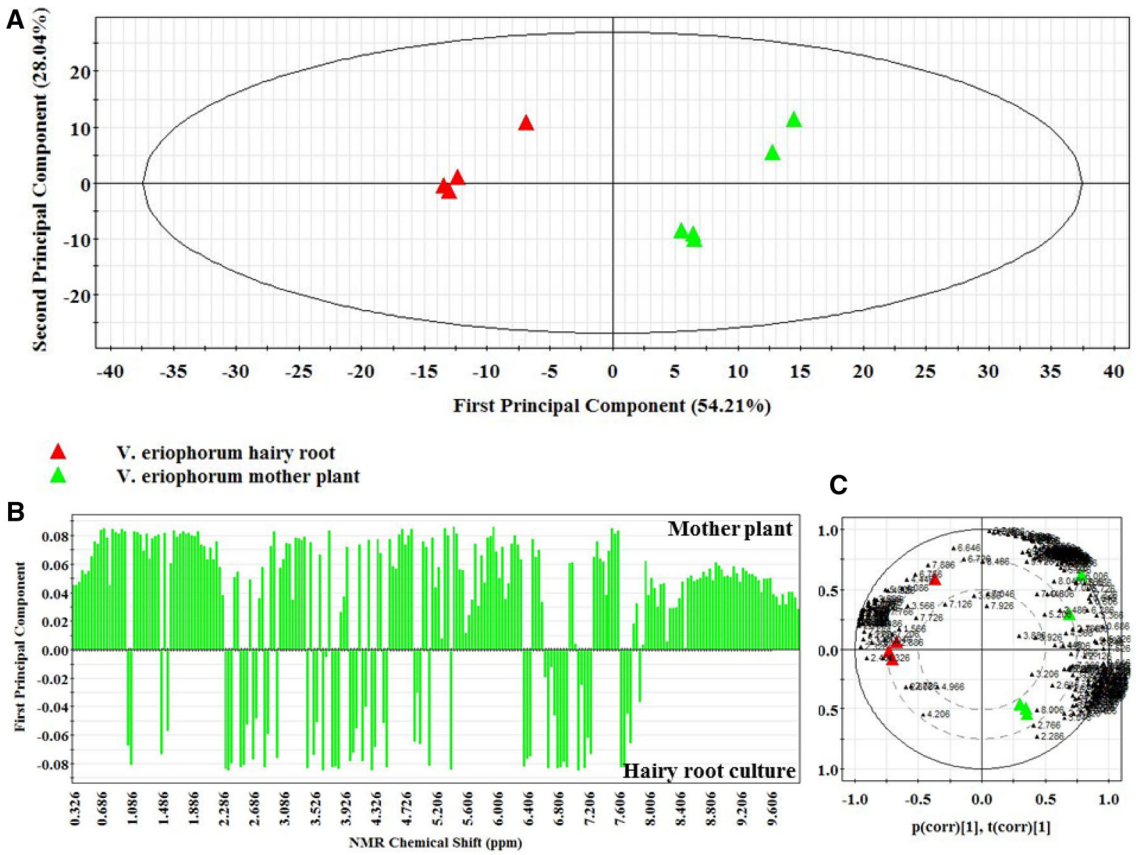

Fig. 5 Principal component analysis (PCA) obtained from proton NMR data of *V. eriophorum* Godr. extracts **(a)**, corresponding loading column plot **(b)** and bi plot **(c)** of intact plants versus. hairy root culture

Martynoside—possessing strong anti-inflammatory properties in terms of inhibiting tumor necrosis faction (TNF)-α—was shown to accumulate in hairy root culture of *Harpagophytum procumbens* (devil's claw) and not in the dedifferentiated cell suspension culture of the same plant species (Gyurkovska et al. 2011).

To examine the differences in diversity of primary and secondary metabolites between mother plant and hairy root culture metabolome, the huge data set from the ^{1}H-NMR were further subjected to multivariate data analysis and PCA in particular (Fig. 5a). A total of 82 % model variance was described with the principal components PC1 and PC2 (Fig. 5a). To determine metabolic alterations of intact plant leaf and hairy root culture metabolome both sample clusters were plotted using PC1. The resultant loading column plot (Fig. 5b) and bi-plot (Fig. 5c) for the PC1 divulged that some NMR signals were highly expressed in hairy root cultures of *V. eriophorum* mostly with aliphatic nature, sugars as well as few signals, belonging to the aromatic molecules (incl. phenylethanoid glycosides). These results are in line with previous report on metabolomics of *V. nigrum* L. hairy roots (Georgiev et al. 2015).

In summary, rare *V. eriophorum* Godr. plants were transformed with *A. rhizogenes*. Further, an NMR-based metabolomics platform was adopted to examine major metabolic differences in *V. eriophorum* as a result of the T-DNA transfer from bacteria. ^{1}H-NMR fingerprinting coupled to the PCA allows identifying the major alterations in metabolome of intact *V. eriophorum* plants and generated hairy root culture. Verbascoside-possessing desirable pharmacological activities for human health—was the most abundant metabolite from phenylethanoids pool and *V. eriophorum* hairy root clones contained several times more of active compounds than the mother plant leaves. Therefore, hairy root culture of *V. eriophorum*

could serve as an attractive producer for sustainable and controlled supply of verbascoside. Moreover, hairy root culture platform offers an immense potential to engineer verbascoside biosynthesis, an opportunity that is barely covered so far.

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References

Alipieva K, Korkina L, Orhan I, Georgiev MI (2014a) Verbascoside—a review of its occurrence, (bio)synthesis and pharmacological significance. Biotechnol Adv 32:1065–1076

Alipieva KI, Orhan IE, Cankaya IIT, Kostadinova EP, Georgiev MI (2014b) Treasure from garden: chemical profiling, pharmacology and biotechnology of mulleins. Phytochem Rev 13:417–444

Dimitrova P, Kostadinova E, Milanova V, Alipieva K, Georgiev MI, Ivanovska N (2012) Antiinflammatory properties of extracts and compounds isolated from *Verbascum xanthophoeniceum* Griseb. Phytother Res 26:1681–1687

Georgiev MI, Weber J (2014) Bioreactors for plant cells: hardware configuration and internal environment optimization as tools for wider commercialization. Biotechnol Lett 36:1359–1367

Georgiev MI, Ali K, Alipieva K, Verpoorte R, Choi YH (2011) Metabolic differentiations and classification of *Verbascum* species by NMR-based metabolomics. Phytochemistry 72:2045–2051

Georgiev MI, Pastore S, Lulli D, Alipieva K, Kostyuk V, Potapovich A, Panetta M, Korkina L (2012) *Verbascum xanthophoeniceum*-derived phenylethanoid glycosides are potent inhibitors of inflammatory chemokines in dormant and interferon-gamma-stimulated human keratinocytes. J Ethnopharmacol 144:754–760

Georgiev MI, Ivanovska N, Alipieva K, Dimitrova P, Verpoorte R (2013) Harpagoside: from Kalahari Desert to pharmacy shelf. Phytochemistry 92:8–15

Georgiev MI, Radziszewska A, Neumann M, Marchev A, Alipieva K, Ludwig-Müller J (2015) Metabolic alterations of *Verbascum nigrum* L. plants and SAArT transformed roots as revealed by NMR-based metabolomics. Plant Cell Tiss Organ Cult 123:349–356

Geu-Flores F, Sherden NH, Courdavault V, Burlat V, Glenn WS, Wu C, Nims E, Cui Y, O'Connor SE (2012) An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis. Nature 491:138–142

Gyurkovska V, Alipieva K, Maciuk A, Dimitrova P, Ivanovska N, Haas C, Bley Th, Georgiev MI (2011) Anti-inflammatory activity of devil's claw in vitro systems and their active constituents. Food Chem 125:171–178

Halabalaki M, Vougogiannopoulou K, Mikros E, Skaltsounis AL (2014) Recent advances and new strategies in the NMR-based identification of natural products. Curr Opin Biotechnol 25:1–7

Kim HK, Choi YH, Verpoorte R (2010) NMR-based metabolomic analysis of plants. Nat Protoc 5:536–549

Kim HK, Choi YH, Verpoorte R (2011) NMR-based metabolomics: where do we stand, where do we go? Trends Biotechnol 29:267–275

Ludwig-Müller J, Jahn L, Lippert A, Püschel J, Walter A (2014) Improvement of hairy root cultures and plants by changing biosynthetic pathways leading to pharmaceutical metabolites: strategies and applications. Biotechnol Adv 32:1168–1179

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nicholson JK (2010) Metabolomics. Nature 463:32

Rai A, Saito K (2016) Omics data input for metabolic modeling. Curr Opin Biotechnol 37:127–134

Runguphan W, Qu X, O'Connor SE (2010) Integrating carbon-halogen bond formation into medicinal plant metabolism. Nature 468:461–467

Saiman MZ, Mustafa NR, Choi YH, Verpoorte R, Schulte AE (2015) Metabolic alterations and distribution of five-carbon precursors in jasmonic acid-elicited *Catharanthus roseus* cell suspension cultures. Plant Cell Tiss Organ Cult 122:351–362

Simmler C, Napolitano JG, McAlpine JB, Chen SN, Pauli GF (2014) Universal quantitative NMR analysis of complex natural samples. Curr Opin Biotechnol 25:51–59

The Plant List. www.theplantlist.org. Accessed on 07 Feb 2016

Tohge T, Fernie AR (2010) Combining genetic diversity, informatics and metabolomics to facilitate annotation of plant gene function. Nat Protoc 5:1210–1227

Turker AU, Camper ND, Gurel E (2001) *In Vitro* culture of common mullein (*Verbascum thapsus* L.). Biol Plant 37:40–43

Velchev V, Kozuharov S, Bondev I, Kuzmanov B, Markova M (1984) Red data book of Bulgaria Plants, vol 1. Bulgarian Academy of Sciences, Sofia, p 334

Zahmanov G, Alipieva K, Simova S, Georgiev MI (2015) Metabolic differentiations of dwarf elder by NMR-based metabolomics. Phytochem Lett 11:404–409