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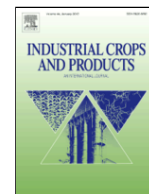


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Agrobacterium rhizogenes-mediated transformation of *Gentiana utriculosa* L. and xanthenes decussatin-1-*O*-primeveroside and decussatin accumulation in hairy roots and somatic embryo-derived transgenic plants

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ABSTRACT

Production of innovative drugs from natural products in controlled conditions plays an important role in modern pharmacology in order to tackle global health challenges. The potential of *Gentiana utriculosa* hairy roots, obtained by transformation with *Agrobacterium rhizogenes* A4M70GUS, as well as that of the shoots regenerated from them via somatic embryogenesis, for xanthone production was investigated. *Gentiana utriculosa* was shown to be a new source of xanthenes, medicinal raw materials for different pharmaceutical applications, among which decussatin has been recently recognized as a prospective hepatoprotective and antiulcer compound. Decussatin and decussatin-1-*O*-primeveroside were detected in both hairy roots and transgenic shoots, while mangiferin, present in nontransformed plants, was not detected. Quantitative HPLC analysis revealed up to 4.5-fold higher decussatin production in hairy roots line 9 compared with the plants from the nature, and this clone was selected. It showed stable growth after more than two years of continuous subcultivation. Cultivation of hairy roots on solid medium was favorable for biomass production, while liquid culture was beneficial for decussatin and decussatin-1-*O*-primeveroside accumulation. Cytokinins kinetin or N⁶-benzyladenine promoted somatic embryo maturation and germination as well as multiplication of obtained plantlets, while active charcoal reduced hyperhydricity. Plants regenerated from selected hairy root line 9 comprised two TL-DNA inserts, as confirmed by Southern blot analysis. They accumulated at least 2.5-fold more decussatin than nontransformed plants. Therefore, these plants could be valuable material to create xanthone high-yielding cultivars of *G. utriculosa*.

1. Introduction

Gentiana utriculosa L. is an annual plant species found in Central Europe, mainly in the Alps, Apennines, Croatia, Balkans and Transylvania (Köhlein, 1991; Tutin, 1972). It is a medium-sized herb, with deep azure-blue flowers, common and widespread in damp meadows and pastures from lowlands up to 2400 m. It has wide distribution in the mountains of Central Serbia (Jovanović-Dunjić, 1977). The aerial parts of *G. utriculosa* are chemically characterized by the presence of xanthone-*O*-glycosides with 1,3,7,8-oxidation pattern along with xanthone and flavone-*C*-glycosides. Decussatin (1-hydroxy-3,7,8-trimethoxyxanthone) and decussatin-1-*O*-

primeveroside (1-*O*-primeverosyl-3,7,8-trimethoxyxanthone), together with mangiferin, gentiacaulein, and lancesin, were detected as the main xanthenes (Janković et al., 2009). Since these compounds display a wide range of different biological activities, e.g. antimicrobial (Šavikin et al., 2009), radioprotective (Menković et al., 2010), antitumor (Isaković et al., 2008), antidepressant (Tomić et al., 2005) and vasodilator (Chericoni et al., 2003), *G. utriculosa* could be considered as a new potential source of medicinal raw materials for different pharmaceutical applications. Unlike the majority gentians, *G. utriculosa* has not been investigated in detail concerning its pharmacological properties and medicinal potential.

Moreover, xanthenes mangiferin and decussatin, present in *G. utriculosa*, are nowadays recognized as promising hepatoprotective

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tive compounds with strong potential to inhibit β -glucuronidase, the enzyme whose increased activity is responsible for carcinogenic transition and liver damage (Karak et al., 2017). Silymarin, a commercial *Silybium marianum*-derived mixture with β -glucuronidase inhibitory activity, used for years to treat liver disorders and related cancers (Kim et al., 1994; Dixit et al., 2007), has poor bioavailability (Dixit et al., 2007), and exhibits some negative effects related to gastrointestinal tract (Pradhan and Girish, 2006). Therefore, the report that decussatin displayed gastric cytoprotective and antisecretory properties in rats makes it an interesting compound for the development of new anti-ulcer drugs (Ateufac et al., 2014). Studies of Ateufac et al., (2007) have also shown the spasmogenic properties of decussatin on isolated gastric smooth muscle, and report that the mechanism of action might be due to an interference with calcium metabolism in smooth muscle.

Results of Ding et al., (2009) also showed that 1-hydroxy-3,7,8-trimethoxyxanthone displayed significant anti-proliferation and apoptosis effect in human leukemia cell line HL-60 in a time and dose-dependent manner. Therefore anticancer pharmacological activity of this xanthone was also suggested with conclusion that safety of dosage should be taken into consideration during the clinical treatment.

Discovering of the innovative drugs from natural products has played important role in modern pharmacology in order to tackle global health challenges (Atanasov et al., 2015). Multiple pharmacological effects of decussatin provide pharmacological support for its usage as a new potential medicine. However, although decussatin has attracted a special attention as natural product of therapeutic interest, its industrial application requires further pharmacological research as well as the suitable strategy for providing its sustainable resources.

The synthesis of biologically active metabolites in plants under the field conditions could be impaired by a number of external influences, causing the alterations in the quality and quantity of targeted metabolites and their production rates. Development of a reliable *in vitro* clonal propagation system could provide multiple benefits, e.g. to maintain stable and efficient secondary metabolite production, to facilitate studies on secondary metabolite production, to enable genetic improvement of plants and germplasm conservation. However, for many *Gentiana* species, protocols for such controlled propagation, leading to the satisfactory production of targeted secondary metabolites, have not yet been established. The protocol for *in vitro* shoot propagation and somatic embryogenesis of *G. utriculosa* has been reported for the first time by Vinterhalter et al., (2016). Despite intensive attempts to optimize tissue culture and regeneration parameters, the authors reported high levels of root and shoot hyperhydricity and necrosis, which subsequently impaired the quality, multiplication rates and growth potential of *in vitro* regenerated shoots, indicating the need for further procedure optimization.

Currently, the establishment of hairy roots by *Agrobacterium rhizogenes*-mediated transformation is considered as a promising approach to overcome the problems and limitations in secondary metabolites production of many plants (Matveeva and Sokornova, 2016; Alpizar et al., 2008; Mitić et al., 2012), as well as to develop superior genotypes with improved production of targeted metabolites e.g. higher xanthone norswertianin-1-*O*-primeveroside-producing clone of *Gentiana dinarica* (Vinterhalter et al., 2015) or selected clone (14-P) of *Picrorhiza kurroa* hairy root with higher potential for iridoid glycosides (picroside I and kutkoside) production (Verma et al., 2007, 2015). Hairy roots typically display fast growth and can be simply maintained on plant growth regulators (PGR)-free medium. They are genetically stable and plant regeneration from them occurs at high frequency in many plant species.

Somatic embryogenesis and plant regeneration from hairy roots have been reported in some valuable medicinal plants, including *Gentiana macrophila* (Wu et al., 2011), *Panax quinquefolium* (Zhao et al., 2012), and *Salvia miltiorrhiza* (Wang et al., 2013). According to the observed capacity of *in vitro* cultures to accumulate secondary metabolites, the establishment and maintenance of *G. utriculosa* hairy roots culture could be a desirable approach to meet increased pharmaceutical demands. Additionally, *G. utriculosa* hairy roots might also be used for clonal propagation of this plant species. Since the plants regenerated from hairy roots frequently have more robust root system than those of non-transformed or natural plants, hairy roots could be also used to improve rooting performances in regenerated plants.

On the basis of increasing medical significance of xanthone compounds, this research was initiated with the aim to establish hairy root cultures of *G. utriculosa* and to investigate their ability to produce xanthone compounds. Xanthone accumulation was also analyzed in transgenic plants derived from somatic embryos that spontaneously regenerated from hairy roots. The elite clone of *G. utriculosa* related to growth, regeneration performances and xanthones production was declared, paving the way for further research with the aim to intensify controlled xanthone production in *G. utriculosa*.

2. Material and methods

2.1. Plant material, bacterial strain and transformation procedure

in vitro-grown shoot cultures of *Gentiana utriculosa* L. (Vinterhalter et al., 2016) were used as material for genetic transformation. Aseptic shoot cultures, established from immature seeds (Vinterhalter et al., 2016), were maintained and multiplied on basal medium (BM) consisting of MS (Murashige and Skoog, 1962) mineral salts, LS (Linsmaier and Skoog, 1965) vitamins, 100 mg L⁻¹ myo-inositol, 2% (w/v) sucrose, solidified with 0.64% (w/v) agar (Torlak Institute of Virology, Vaccines and Sera, Belgrade, Serbia), and supplemented with 0.2 mg L⁻¹ N⁶-benzyladenine (BA). Cultures of nontransformed roots, used for phytochemical analyses, were maintained in liquid BM media lacking agar, on an orbital shaker at 85 rpm. All plant cultures, either prior to or after the transformation, were grown under the controlled optimal cultivation conditions, at 25 °C ± 2 °C and under 16 h light /8 h dark photoperiod provided by cool white fluorescent tubes with a flux rate of 40 $\mu\text{mol s}^{-1} \text{m}^{-1}$ for shoots and 2 $\mu\text{mol s}^{-1} \text{m}^{-1}$ for roots.

For plant tissue inoculation, *Agrobacterium rhizogenes* strain A4M70-GUS, carrying the pRiA4 plasmid (Tepfer and Casse-Delbart, 1987), was used. This strain contains a cointegrative plasmid with a GUS construct integrated into the TL-DNA region of the pRiA4. GUS construct contains *uidA* sequence, coding for β -glucuronidase enzyme, under the 70S promoter (enhancer-doubled 35S CaMV promoter), followed by *nos* polyadenylation sequence. Bacterial cultures were grown on solid YEB (Yeast Extract Beef) media (Van Larebeke et al., 1977) with 100 mg L⁻¹ neomycin at 27 °C in the dark for 3 days. For transformation purpose, overnight bacterial suspensions were made out of one full loop (\emptyset 3 mm) of bacterial culture scraped from a plate and re-suspended in 25 mL of a liquid YEB medium. After overnight incubation on an orbital shaker, *A. rhizogenes* cells were collected by centrifugation at 10,000 x g, and pellet re-suspended in liquid YEB was used for inoculation.

Total of 197 explants, consisting of 15 mm-long shoots, were inoculated by wounding with a needle dipped in bacterial suspension. Inoculated shoots (5 explants) were placed in 100 mL wide-neck Erlenmeyer flasks filled with 40 mL BM for the first 15 days of in-

oculation and then transferred to BM supplemented with 300 mg L⁻¹ Na-cefotaxime (Cef Tolycar®; Jugoremedia, Zrenjanin, Serbia).

Putative transgenic hairy roots were observed emerging three weeks after inoculation. Since most of them appeared to be fragile, each root was detached with the short piece of the initial explant and transferred into plastic Petri dish (Ø 100 mm; Spektar, Čačak, Serbia) filled with 25 mL of solid ½BM (BM with half strength of MS mineral salts and vitamins), supplemented with 500 mg L⁻¹ Cef. Hairy roots from each explant were separated as individual line. Hairy root cultures were maintained under the above described conditions, with the Cef concentration gradually decreasing in the subsequent 5-week long subcultures, down to the Cef-free medium after total of 6 subcultures.

2.2. Histological analysis of somatic embryos development

Material for histological analysis was fixed in FAA (formalin:acetic acid:ethanol 10:5:85 v/v/v), dehydrated in a graded ethanol series, subsequently cleared with xylol and embedded in paraffin wax at 58 °C. Sections 8–10 µm thick were stained with haematoxylin and photographed using a Leitz DMRB photomicroscope (Leica, Wetzlar, Germany).

2.3. Southern blot hybridization

Southern hybridization was performed to confirm the stable integration of T-DNA and to estimate the copy number of the TL-DNA inserted within the genome of *G. utriculosa*. Plant genomic DNA was extracted from the shoots of transformed lines and nontransformed plants following the procedure of Murray and Thompson (1980), while plasmid pRiA4M70GUS DNA was extracted following the procedure of Kado and Liu (1981) and used as a positive control. DNA samples were digested with *Bgl*III endonuclease (Fermentas, Vilnius, Lithuania). One and a half µg of plant DNA samples and 20 ng of plasmid DNA (quantified using a Qubit fluorometer, Invitrogen), were loaded on a 1% agarose gel (Sigma-Aldrich Co.), separated electrophoretically and blotted onto a positively charged nylon membrane (Roche, Indianapolis, IN, USA) by capillary transfer. Fragment of 478 bp was used as a probe for detection of the *rolD* gene, which is located in close proximity to the right TL-DNA border. The probe was labeled by PCR with digoxigenin (DIG)-dUTP (Roche), using forward primer 5'-CCT-TAC-GAA-TTC-TCT-TAG-CGG-CAC-C-3' and reverse primer 5'-GAG-GTA-CAC-TGG-ACT-GAA-TCT-GCA-C-3' (Fig. 3A).

Hybridization was performed in DIG Easy Hyb buffer (Roche) at 48 °C for 16 h. The membrane was then washed 2 x 5 min in each of the following buffers: 2xSSC + 0.1% SDS (Sodium Dodecyl Sulfate) and 1xSSC + 0.1% SDS at 48 °C and 0.5xSSC + 0.1% SDS and 0.1xSSC + 0.1% SDS at 65–68 °C. Hybrids were detected with antidigoxigenin antibody (Roche), visualized with chemiluminescent substrate CDP-Star (Roche) and recorded on X-ray film (Kodak, Rochester, NY).

2.4. Evaluation of hairy roots growth and regeneration potential

To find the optimal conditions for hairy roots growth, elongated roots of three individual lines (4, 9 and 12) with confirmed integration of T-DNA, were further cultivated either in liquid or on solid ½BM. For each individual line, hairy roots at initial weight of 400 mg were cultivated in Erlenmeyer flasks with liquid medium or in plastic Petri dishes with solid medium. After 35 days, the fresh weight (FW) of roots was measured and the growth index (GI) was calculated as: $GI = (\text{final FW} - \text{initial FW}) / \text{initial FW}$.

The roots were then left to dry at room temperature, and the dry weight (DW) was recorded. On almost all tested roots, grown either in liquid or on solid media, spontaneous somatic embryogenesis occurred during these 35 days. The number of developed somatic embryos was determined under a stereomicroscope and presented as mean values per Erlenmeyer flask or Petri dish. For the solid medium, the effect of active charcoal (AC) at the concentration of 1 mg L⁻¹ on all the abovementioned parameters of hairy roots growth was additionally tested.

2.5. The effect of plant growth regulators on somatic embryo development

Somatic embryos (SEs) regenerated from transgenic roots maintained either in liquid or on solid ½BM, were detached and transferred onto the solid BM supplemented with 1 mg mL⁻¹ AC without plant growth regulators (PGR) or with 0.1 mg L⁻¹ N⁶-furfuryl aminopurine (kinetin, Kin) or 0.2 mg L⁻¹ BA. After 35 days, the number of necrotic, hyperhydric, and healthy SEs germinated into plantlets, was recorded and presented as the percentage of total regenerated embryos.

In order to establish in vitro shoot cultures, viable germinated SEs were subsequently transferred onto the same solid BM media where hairy roots regenerating SEs were previously maintained. After 5 weeks, the number of axillary buds per plantlets were scored and presented as mean multiplication ratio (= main shoot + axillary buds for each group of plantlets obtained from SEs germinated on different media).

2.6. Extraction and HPLC analysis of xanthenes

Xanthone identification and content determination were performed in extracts from in vitro-derived plantlets and wild growing plants, collected from two natural habitats located on Stara Planina mountain (~ 1100 m) in eastern Serbia (Loc 1) and Divčibare (~ 980 m) in western Serbia (Loc 2). The samples were air-dried at room temperature and ground to fine powder using a mortar and a pestle. The obtained powder (500 mg) was extracted with 10 mL of methanol in ultrasonic bath for 20 min. After sonication, extraction was continued by maceration in the dark at room temperature, for 48 h. The extracts were filtered through Whatman filter paper No.1 into 10-mL volumetric flasks and adjusted to the volume with methanol. Prior to HPLC analysis, extracts were filtered through 0.45-µm nylon syringe filters (Captive Econo Filters, 13 mm, Agilent Technologies).

Identification and quantification of xanthenes were performed by chromatographic analysis (Agilent series 1100 HPLC instrument, with a diode array detector) on a reverse phase Zorbax SB-C18 (Agilent) analytical column (150 mm x 4.6 mm i.d., 5 µm particle size) thermostated at 25 °C. The mobile phase consisted of solvent A (1%, v/v solution of orthophosphoric acid in water) and solvent B (acetonitrile, J.T. Baker, Deventer, The Netherlands). The separation of the components was performed using the gradient elution as follows: 98-90% A 0–5 min, 90% A 5–10 min, 90-85% A 10–13 min, 85% A 13–15 min, 85-70% A 15–20 min, 70-40% A 20–24 min, 40-0% A 24–28 min. Sample injection volume was 5 µL. Column elution was monitored at 260 and 320 nm, and the flow rate was 1 mL min⁻¹. Standards of xanthenes decussatin-1-O-primeveroside and decussatin were previously isolated in our laboratory from aerial parts of *G. utriculosa* (Janković et al., 2009). Mangiferin was purchased from Sigma-Aldrich Inc., Germany/USA. Standard solutions for HPLC were prepared by dissolving xanthone compounds in methanol. Quantification was performed using calibration curves in external standard method. All experiments were

repeated at least two times. The results are presented as mg g^{-1} of DW of extracts.

2.7. Statistical analysis

The effects of genotype and nutrient media type or composition on parameters of transgenic hairy roots growth (GI, DW and number of SEs) and xanthone production were evaluated using two-way analysis of variance (ANOVA). Differences between the corresponding means were separated using Fisher's LSD test at $P \leq 0.05$. Additionally, Student's *t*-test was employed to compare the differences in xanthone content between nontransformed controls and transgenic lines (for each line and each growing condition vs. controls). For multiplication ratio comparison within each transgenic line, one-way ANOVA with nutrition media as a factor has been employed for both liquid and solid medium separately. Statistical analyses were performed using SAS software (SAS Institute, 2002; SAS/STAT, ver. 9.00. SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Induction of *Gentiana utriculosa* hairy roots

Three weeks after total of 197 *in vitro*-grown *G. utriculosa* shoot explants were inoculated with *A. rhizogenes* strain A4M70GUS, the first hairy roots appeared at the wounded sites (Fig. 1A), while 45 days after inoculation 21 of them (10.66%) produced extensive, fibrous hairy roots, mostly without lateral branches and root hairs. The majority of detached roots was fragile, grew slowly and eventually became necrotic during cultivation on media supplemented with antibiotic Cef. Concomitantly, proliferated calli with green globules, as indicators of somatic embryo regeneration, were observed on some hairy roots (Fig. 1B).

Histological analysis of hairy roots indicated indirect somatic embryogenesis (Fig. 2). On the surface of hairy roots a parenchymatic callus tissue developed in which meristematic zones differentiated. Meristematic cells differed from surrounding parenchyma cells by dense, intensively stained cytoplasm and conspicuous nucleus with nucleolus (Fig. 2A,B). Anticlinal divisions of single cell in the surface layer indicated a unicellular origin of SEs (Fig. 2A). Proembryonic structures were observed on the surface of meristematic zones. SEs were separated with protoderm from neighboring tissues and did not have connections with vascular elements of roots (Fig. 2B, C). SE development was not synchronized since embryos at different developmental stages were simultaneously present in the same section (Fig. 2D, E). In bipolar structures, root and shoot meristematic cells at opposing poles are smaller and more in-

tensively stained than the neighboring cells. In later torpedo and cotyledonary stage somatic embryos provascular elements are visible (Fig. 2D, F).

After 6 subsequent subcultures, when Cef was completely omitted from the nutrient media, 5 hairy root lines (1, 4, 9, 12, and 13) (2.54%) continued to grow. All these hairy root lines were subjected to molecular analyses to confirm their transgenic nature.

3.2. Southern blot detection of transgenes

Southern blot hybridization revealed the presence of 2–5 copies of the TL-DNA within the genomes in all 5 transformed lines (Fig. 3). All obtained fragments hybridizing with the probe were longer than the minimal expected size (4230 bp), indicating that all TL-DNA copies were of the full length. Positive control produced a band of approximately 9 kb, while no signal was observed in nontransformed control.

The highest number of copies was recorded in lines 13 and 12 (4 and 5, respectively), while two copies of TL-DNA were detected in lines 1, 4 and 9 (Fig. 3B).

3.3. Phenotype of transgenic hairy roots and spontaneous somatic embryo regeneration

Roots of transgenic lines exhibited significant phenotypic alterations and differed in their growth and regenerating potential both in liquid and on solid media (Fig. 4). When maintained on solid $\frac{1}{2}$ BM, only hairy roots of the line 12 exhibited some of the typical "hairy root syndrome" characteristics: they were elongated, thick, with numerous lateral roots and with minor callus formation (Fig. 4A). By contrast, hairy roots of lines 4 and 9 were brittle, shorter and thicker, with weak lateral branching, and with more intensive callus formation, containing somatic embryos. Hairy roots of line 13 were mostly distinguished from the common hairy root phenotype, comprising thick and poorly growing roots, almost totally covered with large calli containing a lot of SEs, mainly clustered and hyperhydric, and providing limited amounts of available material for some of the following analyses.

The observed hairy root phenotype manifestations were further investigated on roots maintained in parallel in liquid and on solid $\frac{1}{2}$ BM (Fig. 4B). Type of the media and genotype, individually or conjoined, influenced the growth index, dry weight or number of regenerated SEs in different ways (Table 1). Solid media were less favorable for growth of hairy root line 4, but these roots had a higher dry weight than those maintained in liquid media. The opposite was observed for roots of line 9, where solid media facilitated growth, while the roots of line 12 grew equally well on both media

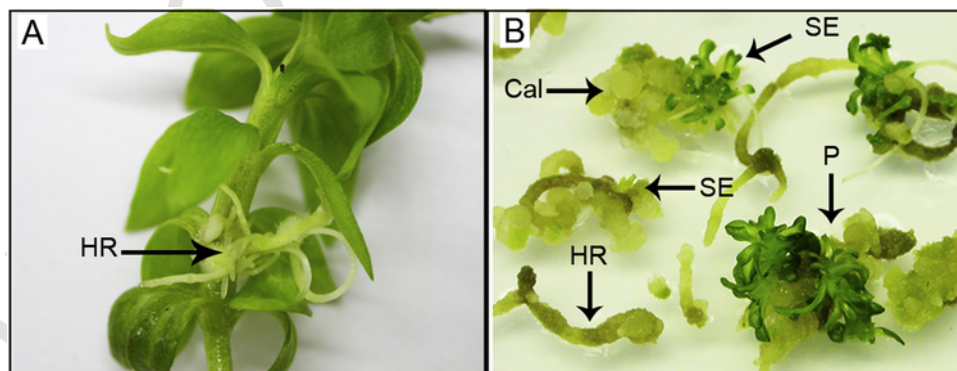


Fig. 1. *Agrobacterium rhizogenes* A4M70GUS-mediated transformation of *Gentiana utriculosa*. A) An *in vitro*-grown shoot explant with emerged hairy roots (HR) three weeks after inoculation. B) Callus (Cal) formation, spontaneous somatic embryo (SE) regeneration, and SE-germinated single plantlets (P) could be seen during the cultivation of detached HR on media supplemented with cefotaxime.

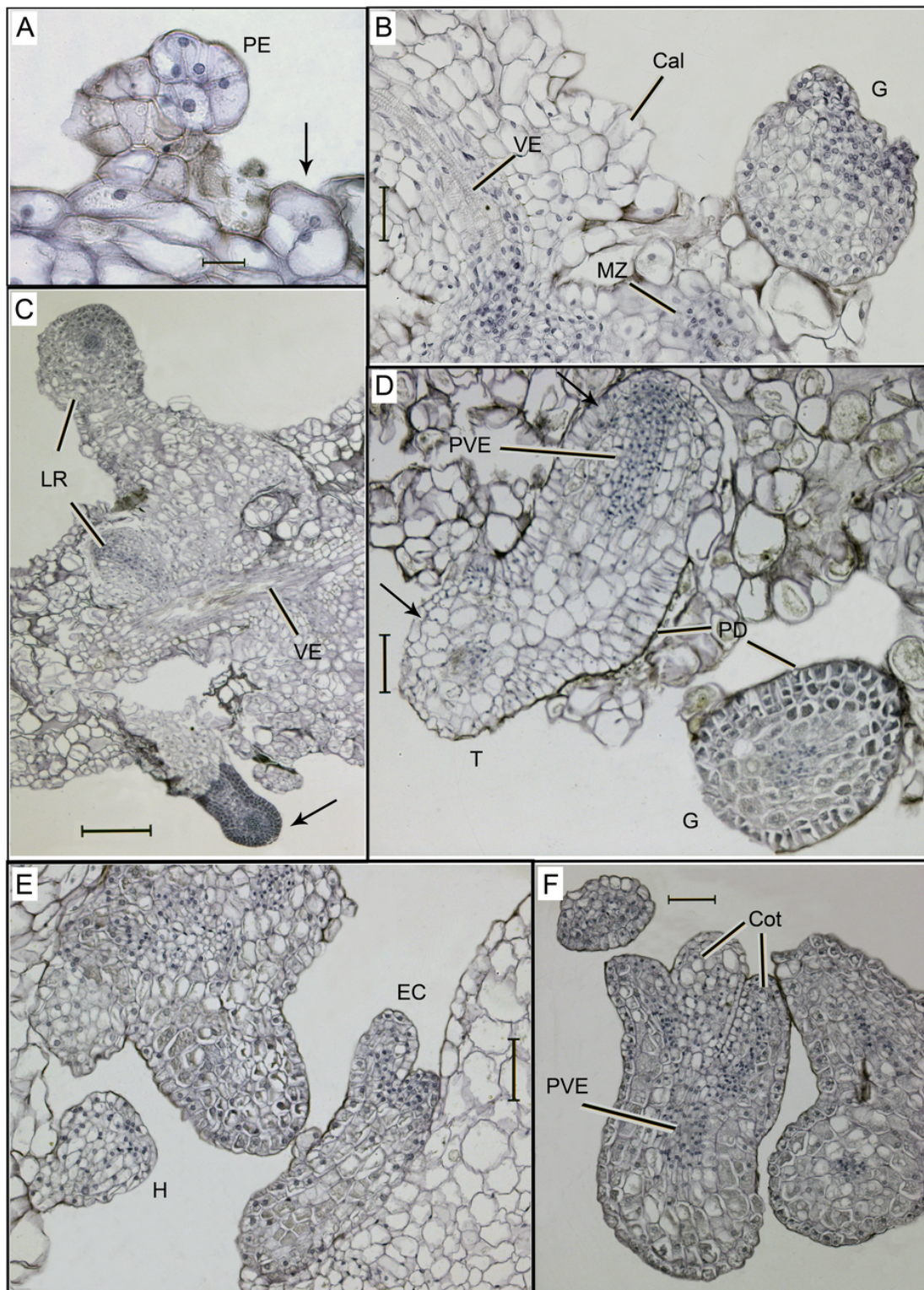


Fig. 2. Histological analysis of somatic embryogenesis from hairy roots of *Gentiana utriculosa*. (A) Anticlinal division of single cell in the surface layer of explant (arrow) and a five-cell proembryo (PE); bar = 20 μm . (B) and (C) Longitudinal section of a hairy root with differentiated vascular elements, callus, and meristematic zones, showing (B) somatic embryo at globular stage; bar = 50 μm , and (C) lateral roots and somatic embryo at cotyledonary stage (arrow); bar = 200 μm . (D) Globular and torpedo somatic embryos with protoderm on the surface. At torpedo stage shoot and root poles (arrows) and provascular elements are visible; bar = 100 μm . (E) Heart and early cotyledonary somatic embryos; bar = 80 μm . (F) Cotyledonary somatic embryo with cotyledons and provascular elements; bar = 80 μm . Proembryo (PE), provascular elements (PVE), vascular elements (VE), callus (Cal), protoderm (PD), meristematic zones (MZ), globular somatic embryos (G), torpedo somatic embryos (T), heart somatic embryos (H), early cotyledonary somatic embryos (EC), lateral roots (LR), (Cot) cotyledon.

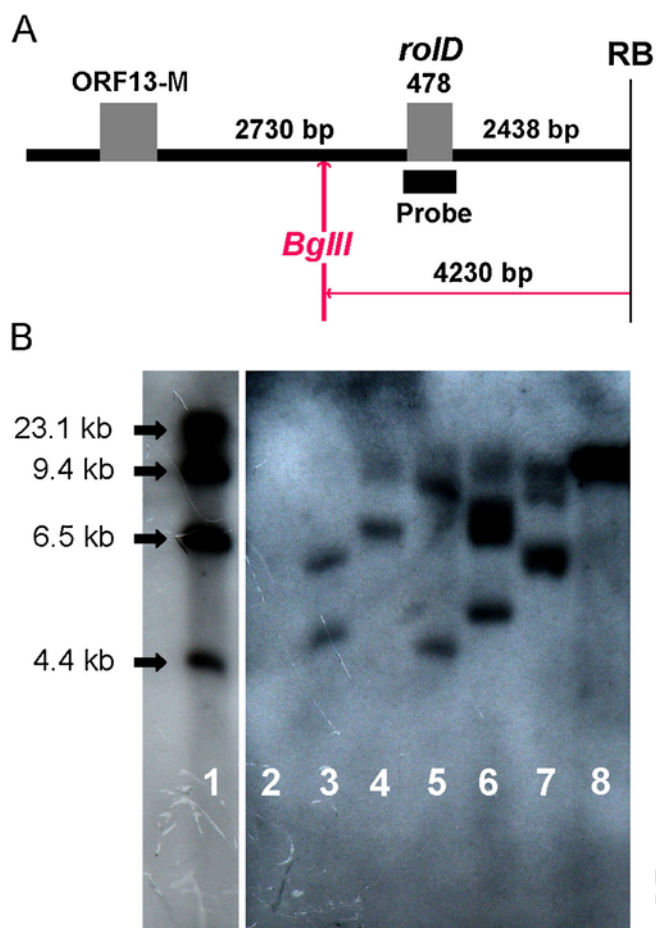


Fig. 3. Southern blot analysis of genomic DNA of putative transformed lines of *Gentiana utriculosa*, performed for TL-DNA copy number estimation. (A) A restriction map of the right part of the TL-DNA depicting the position of the *roID* probe. (B) Genomic DNA was digested with *BglII* endonuclease and hybridized to the *roID* probe. Lane 1: DNA molecular weight marker II (Roche Diagnostics Corporation) labelled with digoxigenin. Lane 2: Negative control, genomic DNA isolated from nontransformed plants. Lanes 3-7: Genomic DNA isolated from shoots of transformed lines 1, 4, 9, 12, and 13, respectively. Lane 8: Positive control, plasmid pRiA4M70GUS DNA digested with *BglII* endonuclease.

types. Hairy roots of line 9 showed the highest regeneration potential with the highest average number of SEs (27 per Erlenmeyer flask in liquid or 15 per Petri dish on solid medium).

The addition of AC into solid medium (Fig. 4C) improved root growth only in line 4, but without positive influence on root dry weight, and with negative impact on SE regeneration potential. Having no effect on GI and DW, AC addition halved the number of SEs in line 9.

3.4. The effect of plant growth regulators on somatic embryo development

During the cultivation of hairy roots in liquid or on solid $\frac{1}{2}$ BM, spontaneous regeneration of SEs, either directly from the roots, or indirectly from calli formed on root sections, was observed. This process asynchronously occurred during the cultivation, thus different stages of SE development (from globular stage embryos to germinated plantlets) were observed on the same hairy root. A number of regenerated SEs displayed certain morphological malformations due to necrosis or hyperhydricity regardless of the type of the maintenance medium used (Fig. 5A).

The percentage of necrotic, hyperhydric and healthy SEs, as well as shoot regeneration potential depended on both hairy root line and the type of previously used maintenance nutrient media (Fig. 5B).

SEs isolated from the hairy roots cultivated in liquid BM displayed high percentage of necrosis, ranging from 56% in line 13 to nearly 80% in line 4 (Fig. 5B). Hairy root line 9 gave rise to the highest number of normal SEs (22.5%), which developed into healthy plantlets during the next 5 weeks. On the other hand, SEs isolated from hairy roots cultivated on solid BM, exhibited significantly less morphological malformations than those grown in liquid medium (Fig. 5B). Only in line 13, the majority of SEs succumbed to necrosis. Although hyperhydricity of SEs occurred in all lines, the number of healthy looking SEs increased in comparison to liquid media. The highest number of healthy looking SEs was recorded in line 4 (40%), as opposed to just 5% of SEs in line 13.

To improve SE stability, both liquid and solid types of BM were supplemented with 0.1 mg L^{-1} KIN or 0.2 mg L^{-1} BA. However, in liquid media, the number of necrotic SEs increased in 3 out of 4 analyzed lines (4, 12 and 13), while the number of healthy looking SEs decreased. Additional enrichment of cytokinin-containing media with AC caused the reduction of necrosis in lines 4 and 12. The addition of cytokinins to solid media slightly affected distribution pattern of SE types, except for the line 4, where the addition of BA reduced the number of necrotic SEs (10% compared with 45% on BM), as well as the number of healthy SEs (10% compared with 40% on BM).

During the 5-week long cultivation, most of the healthy looking SEs germinated into plantlets (Fig. 5C) that were detached and individually cultivated on solid BM supplemented with AC and cytokinins. In general, these regenerated *G. utriculosa* transgenic plantlets displayed low multiplication ability in all analyzed lines (Fig. 5D). A few plantlets developed from the SEs obtained in liquid cultures multiplied sporadically. Multiplication pattern of plantlets, derived from SEs developed from hairy roots cultivated on solid media, was similar to that for SEs obtained from hairy roots cultivated in liquid medium. Mainly, the highest number of lateral buds was observed in lines 4 and 12 maintained on BM + KIN + AC (Fig. 5D).

Since KIN promoted development of healthy looking plantlets, it was used for further maintenance of *G. utriculosa* transgenic shoots. Subcultivation was performed in 5-week intervals on BM with 0.1 mg L^{-1} KIN and supplemented or not with 1 mg L^{-1} AC (Fig. 6A), resulting in healthy looking rooted plants (Fig. 6B) that frequently flowered under *in vitro* conditions (Fig. 6C). Shoots often displayed different phenotypic appearance within the same line (Fig. 6D). All lines showed stable growth and multiplication for more than two years of *in vitro* culturing on abovementioned nutrition medium (data not shown). The mean multiplication ratio for lines 4, 12 and 13 ranged between 1.2–1.5, while plantlets of line 9 showed the highest mean multiplication ratio of 2.1 over this period and were with the most stable phenotype characteristics.

3.5. Xanthone production in hairy roots and regenerated plants

Decussatin-1-*O*-primeveroside (2) and its corresponding aglycon decussatin (3) were found to be dominant xanthones in five-week-old hairy root lines 4, 9, 12, and 13, grown either in liquid or on solid $\frac{1}{2}$ BM, as well as in the control nontransformed (NT) roots (Fig. 7). HPLC analysis also revealed the presence of xanthone-C-glucoside mangiferin (1), gentiakoichianin-1-*O*-primeveroside (4), 1,8-dihydroxy-3-methoxy-7-*O*-primeveroside (5) and aglycons gentiakoichianin (6) and gentiacaulein (7), mostly in lower amounts.

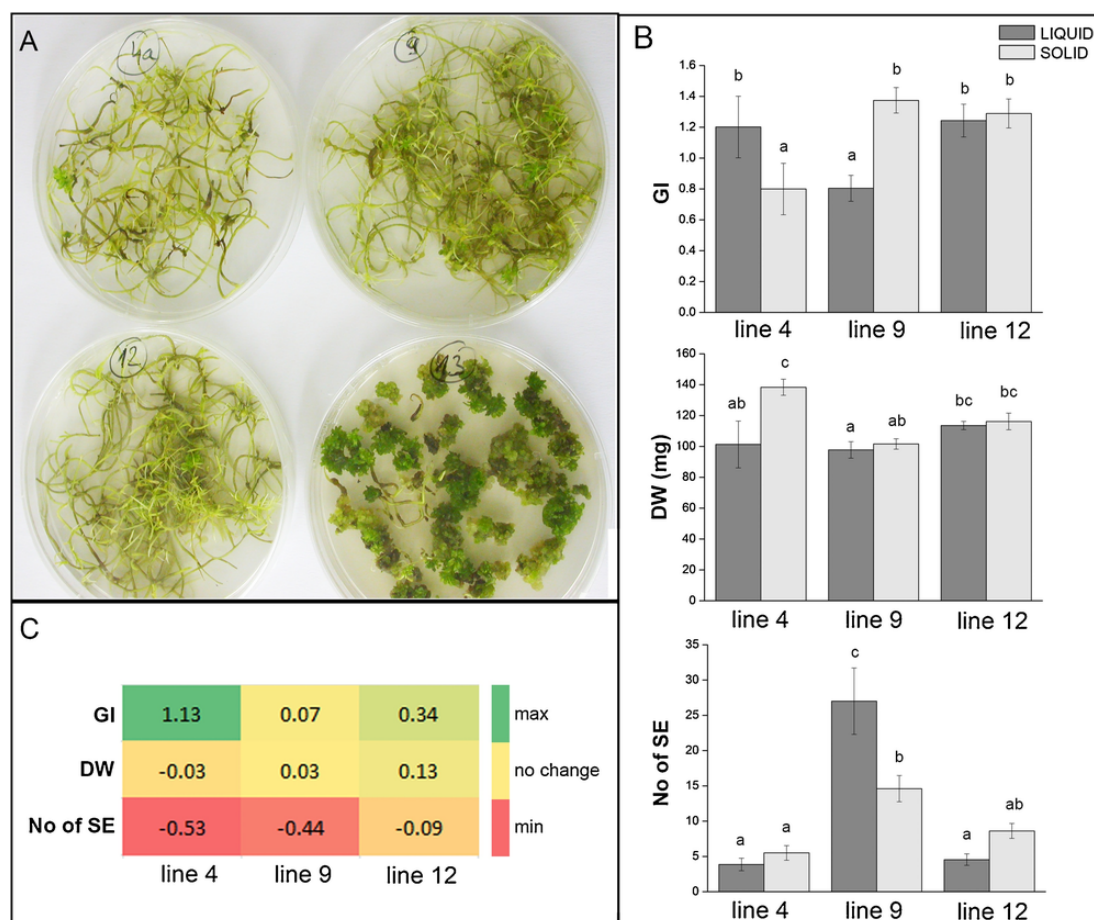


Fig. 4. Phenotypic appearance and regeneration potential of *Gentiana utriculosa* hairy root lines. (A) Phenotypic alterations and differences in growth potential of hairy roots maintained on $\frac{1}{2}$ BM solid medium after 5 weeks of cultivation. (B) Growth and regeneration potential of hairy roots maintained in parallel in liquid and on solid $\frac{1}{2}$ BM analyzed through growth index (GI), dry weight (DW) and number of regenerated somatic embryos (No of SE) after 5 weeks of cultivation. Results are presented as mean number per Erlenmeyer flask (for liquid) or Petri dish (for solid media). Values denoted with the same letters are not significantly different at $P \leq 0.05$ level according to Fisher's LSD test. (C) Effects of active charcoal (AC) on GI, DW and No of SE for roots maintained on solid media, were presented as relative values normalized to corresponding values for roots from media without AC (set as 1).

Table 1

Two-way ANOVA tables for the effect of nutrient media type, genotype and their combination on growth index (GI), dry weight (DW) and number of developed somatic embryos (SEs) of transgenic *Gentiana utriculosa* hairy root lines 4, 9 and 12.

	df	Mean square	F	P
<i>GI</i>				
type of medium	1	0.122	0.5213	0.472
genotype	2	0.593	2.5225	0.0854
type of medium x genotype	2	1.812	7.7170	0.0007
<i>DW</i>				
type of medium	1	2650.5	5.5811	0.0000
genotype	2	2395.4	5.0440	0.0208
type of medium x genotype	2	1098.6	2.3130	0.1061
<i>Number of SEs</i>				
type of medium	1	119.5	0.9624	0.3289
genotype	2	2736.0	22.0353	0.0000
type of medium x genotype	2	746.0	6.0082	0.0034

Their chemical structures and chromatographic data are shown in Fig. 7.

The content of dominant xanthones in hairy roots differed among individual hairy root lines and between two different types

on nutrient media for the roots of the same line, according to two-way ANOVA (Table 2). The most abundant xanthone in all roots, including transgenic lines, NT controls and plants from the nature, was decussatin-1-*O*-primeveroside (Fig. 7). Its amount ranged from 4.3 to 23.7 mg g⁻¹ DW and significantly differed among transgenic lines under the influence of nutrient media types, genotype and their interaction. The roots cultivated in liquid medium produced significantly higher amounts of decussatin-1-*O*-primeveroside compared with those grown on solid medium, with the highest content determined in lines 9 and 13. Additionally, decussatin-1-*O*-primeveroside accumulation was significantly different in all analyzed lines on both nutrient media (except for line 13 in liquid medium) compared with NT according to *t*-test. However, only roots of line 9 cultivated in liquid medium accumulated considerably enhanced amount of this xanthone (23.74 mg g⁻¹ DW) in comparison to NT controls (18.23 mg g⁻¹ DW). The content of decussatin-1-*O*-primeveroside in roots of plants collected from the nature was similar for two distinct localities (10.35 and 10.26 mg g⁻¹ DW for Loc 1 and Loc 2, respectively) and lower than in both in vitro-grown NT roots and transgenic roots of line 9.

Similar pattern of accumulation has been observed for aglycone decussatin (Fig. 8). The highest accumulation of decussatin was again observed in lines 9 and 13, maintained in liquid media (6.10 and 3.64 mg g⁻¹ DW, respectively). These concentrations were significantly higher compared with control roots (1.81 mg g⁻¹ DW, ac-

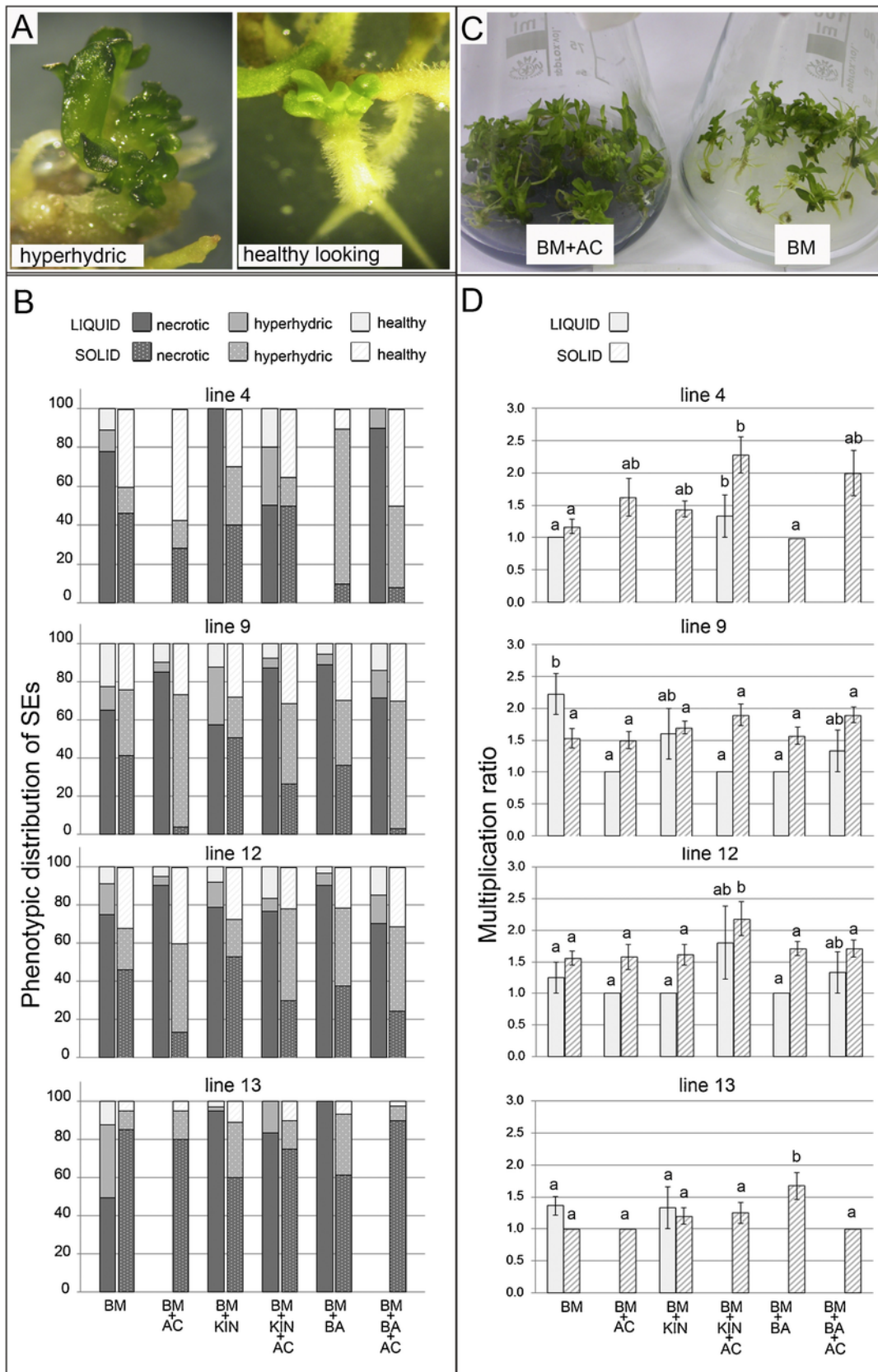


Fig. 5. Spontaneously regenerated SEs on *Gentiana utriculosa* transgenic hairy roots (A) frequently showed necrosis or hyperhydricity in contrast to healthy looking appearance. (B) Number of necrotic, hyperhydric and healthy looking SEs detached from roots grown in liquid or on solid media, presented as a percentage of total number of SEs after 5 weeks of subsequent subculture on solid basal media (BM), or BM supplemented with either 0.1 mg L⁻¹ kinetin (KIN) or 0.2 mg L⁻¹ N⁶-benzyladenine (BA), with the addition of 1 mg mL⁻¹ active charcoal (AC). (C) Germination of healthy looking somatic embryos

of line 4 maintained for the next 35 days on BM or BM + AC showed differences in multiplication. (D) Multiplication ratio for germinated somatic embryos of all analyzed lines has been presented per plantlet for each group as mean values \pm standard errors. Values for multiplication ratios for each transgenic line were subjected to one-way ANOVA with nutrition media composition as factor for liquid or solid medium separately, and compared by Fisher's LSD test at $P \leq 0.05$.

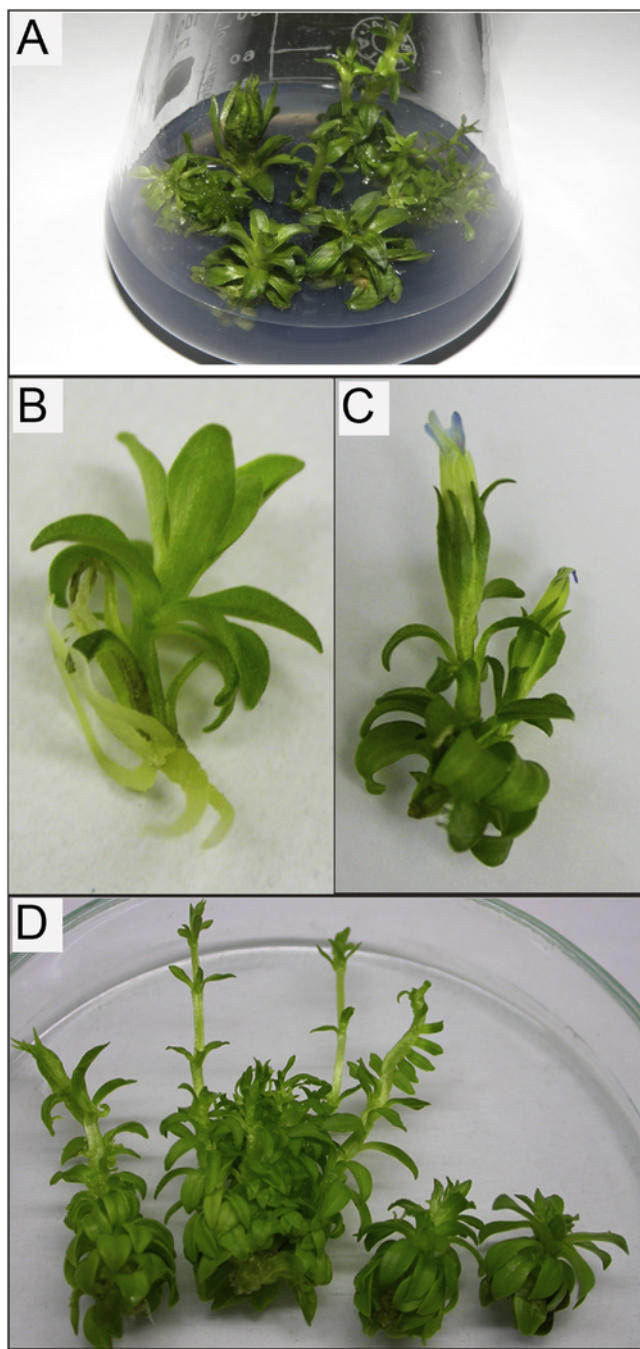


Fig. 6. *In vitro* cultures of transgenic *Gentiana utriculosa* line 9. (A) Plantlets maintained on KIN- supplemented BM with AC giving healthy looking rooted plants (B). (C) Flowering frequently occurred under *in vitro* conditions. (D) Plantlets of the same line often displayed morphological differences.

according to *t*-test). Except for the roots of line 9 cultivated on solid media that contained high level of decussatin ($3.14 \text{ mg g}^{-1} \text{ DW}$), decussatin content in all other samples was similar to its level in NT roots. Although mangiferin has been detected in roots of plants collected from both localities, this xanthone was detected in small quantities ($\leq 0.1 \text{ mg g}^{-1} \text{ DW}$) only in line 13 grown on solid media.

Xanthone content was also analyzed in five-week-old shoots of transgenic lines, NT control shoots and plants collected from the nature (Fig. 9). Decussatin-1-*O*-primeveroside, decussatin and mangiferin were detected and their content was found to be influenced by transgenic nature of lines, nutrient media and their interaction (Table 3). Decussatin-1-*O*-primeveroside was also dominant compound (Fig. 9) in all analyzed shoot samples. However, the content of decussatin-1-*O*-primeveroside was reduced compared with NT in almost all transgenic shoots with the exception of line 9 shoots, grown on PGR-free BM, which produced slightly higher amount of this compound ($8.13 \text{ mg g}^{-1} \text{ DW}$) than NT control ($7.40 \text{ mg g}^{-1} \text{ DW}$). Similar levels of decussatin-1-*O*-primeveroside were detected in both samples from the nature.

The shoots of line 9 grown on the same nutrient media also produced the highest amount of decussatin, exceeding $4.54 \text{ mg g}^{-1} \text{ DW}$ (Fig. 9). In shoots of all other transgenic lines, as well as in NT controls, its amount was below $2.10 \text{ mg g}^{-1} \text{ DW}$. Decussatin was also present in both samples collected from the nature with the higher content detected for Loc 1, which was similar to that recorded in the most productive shoot line 9 grown on PGR-free BM.

Contrary to hairy roots where mangiferin was detected only in line 13, this compound was detected in shoots of all transgenic lines (Fig. 9) but at lower levels than in NT controls. Among transgenic lines, the shoots of line 12 cultured on cytokinin-free BM produced the highest amount of this compound ($2.06 \text{ mg g}^{-1} \text{ DW}$). Mangiferin was the only analyzed xanthone whose content was higher in plants from the nature than in any of the *in vitro*-grown shoots.

4. Discussion

Although many factors can influence the effectiveness of *A. rhizogenes*-mediated transformation, the relationship between virulence ability of particular *A. rhizogenes* strain and plant genotype specificity is crucial for successful transformation (Opabode, 2006). Hairy root cultures of *G. utriculosa* were established using A4M70GUS strain, which was an appropriate vector for transformation of many *Gentiana* species, enabling transformation efficiency from 6.12% in *G. dinarica* (Vinterhalter et al., 2015) to even 63.4% in *G. cruciata* (Momčilović et al., 1997). Numerous *G. utriculosa* hairy root lines were obtained after inoculation with *A. rhizogenes*, five of which (1, 4, 9, 12, and 13) survived and gave rise to hairy root cultures. Therefore, with 2.54% transformation efficiency, *G. utriculosa* could be characterized as recalcitrant-to-transform species.

The high growth capacity and rapid biomass increment is the common characteristic of hairy root cultures in the majority of plant species. Thus, the most productive hairy root lines of *G. dinarica* and *G. punctata*, obtained using A4M70GUS strain, increased their biomass 7.8-fold (Vinterhalter et al., 2015) and 3.4-fold (Vinterhalter et al., 1999, 2000), respectively. In other *Gentiana* species, transformed with different *A. rhizogenes* strains, hairy root biomass increment ranged from 18.7-fold in *G. macrophylla* (Zhang et al., 2010) to 36-fold in *G. scabra* (Huang et al., 2014). However, the low growth index observed in liquid culture system of *G. utriculosa* (up to 1.2 after 35 days of cultivation) also pointed out at the recalcitrant nature of this species.

Additionally, hairy root cultures of *G. utriculosa* differed in growth potential and phenotypic characteristics. Although transgenic nature of these hairy root clones was confirmed by Southern blot analysis, they were characterized by relatively inferior

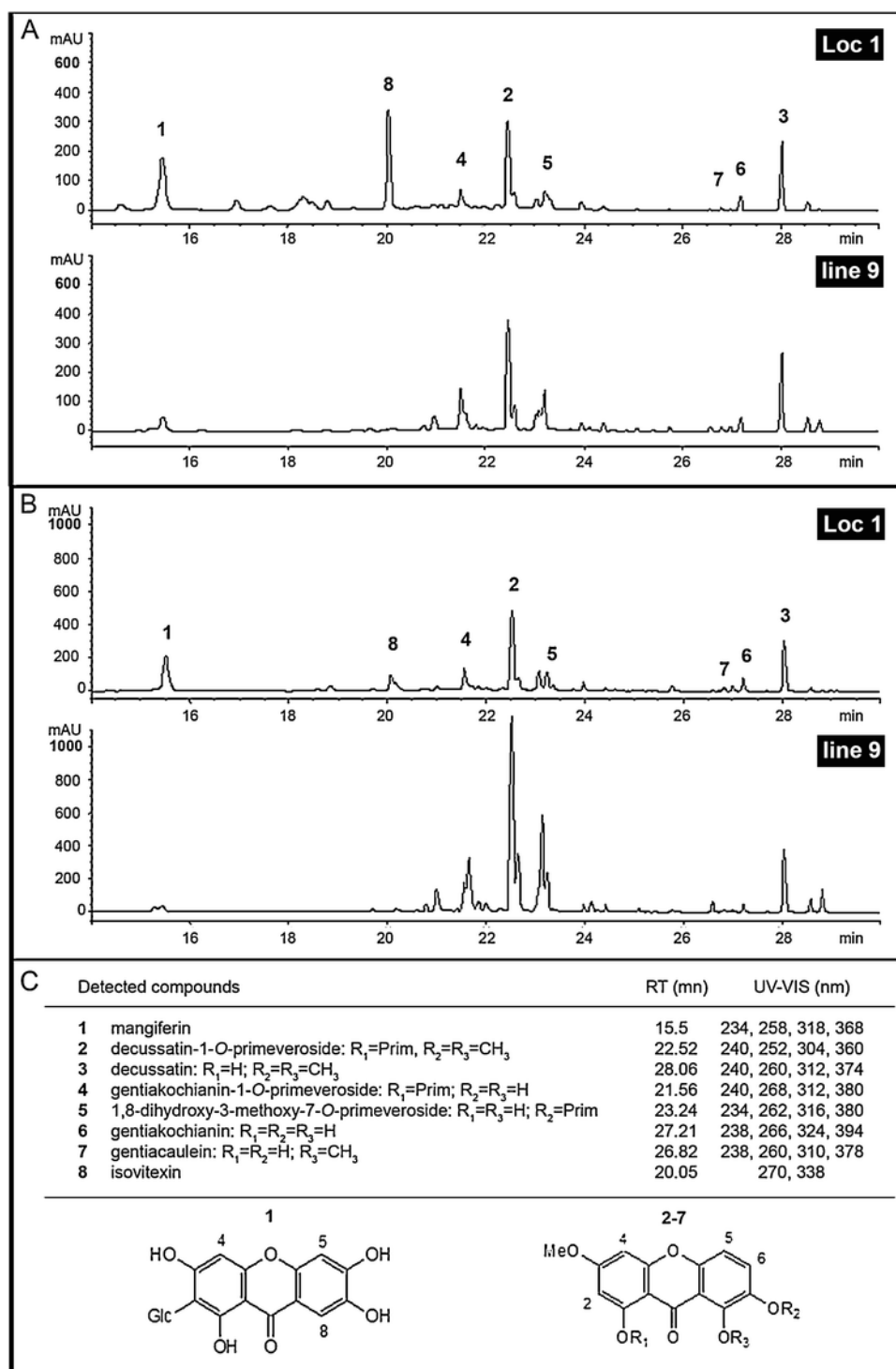


Fig. 7. HPLC profiles of *Gentiana utriculosa* methanol extracts of plants collected from nature (Loc 1) and transgenic line 9 grown *in vitro*. Chromatograms of analyzed shoots (A), roots (B), and chemical structures with chromatographic data for detected compounds (C) were presented.

growth and atypical hairy root phenotype, considering the weak lateral branching (except for line 12) and root hairs. However, this is not exceptionality of *G. utriculosa* since the similar phenotypic characteristics were observed in hairy root clones of other *Gentiana* species (Vinterhalter et al., 1999).

The hairy root cultures have become fashionable biotransformation system for generation of new natural product derivatives with improved pharmaceutical properties (Srivastava et al., 2017). Hairy root cultures substrates of some medicinal plants, such

as *Panax ginseng* (Kawaguchi et al., 1990; Ge et al., 2014) and *Rhodiola kirilowii* (Grech-Baran et al., 2014), have been explored for the bio-conversion into the molecules of still improved pharmaceutical properties. Phytochemical analyses revealed that the secondary metabolite levels accumulated in hairy roots are often comparable to or greater than that of intact plants (Sevón and Oksman-Caldentey, 2002). *G. utriculosa* hairy roots displayed slightly modified xanthone metabolic profile compared with plants collected from the nature, related to mangiferin absence or its undetectable

Table 2

Two-way ANOVA tables for the effect of nutrient media type, genotype and their combination on xanthone content in transgenic *Gentiana utriculosa* hairy root lines 4, 9 and 12.

	df	Mean square	F	P
<i>Decussatin-1-O-prim</i>				
type of medium	1	555.57	30.755	0.00003
genotype	3	41.87	2.3178	0.10993
type of medium x genotype	3	43.546	2.4106	0.1105
<i>Decussatin</i>				
type of medium	1	2.175	8.2749	0.01004
genotype	3	12.626	48.036	0.0000
type of medium x genotype	3	4.947	18.8207	0.0000

amounts in transgenic roots. On the other hand, hairy roots of line 9 produced up to 2.5-fold higher amount of decussatin-1-O-primeveroside and decussatin, compared with plants collected from two localities on Serbian mountains Stara planina and Divčibare, as well as significantly higher amounts of these compounds compared with non-transformed in vitro-maintained roots. Given that new findings suggest the beneficial pharmacological activities of decussatin (Ateufac et al., 2014; Karak et al., 2017), highly regenerating line 9 could be considered as a valuable tool for large scale production of this compound in a sustainable manner without environ-

mental fluctuations (Veena and Taylor, 2007; Banerjee et al., 2012).

Hairy roots can regenerate whole plants, which has been reported in some medicinal plants including *Panax ginseng* (Yang and Choi, 2000), *Catharanthus roseus* (Choi et al., 2004), *Crotalaria spectabilis* (Ohara et al., 2012), and *Salvia miltiorrhiza* (Wang et al., 2013). In *G. utriculosa*, spontaneous somatic embryos regenerated immediately after the hairy roots have been isolated from explant tissue and cultivated individually. The regeneration of somatic embryos from hairy roots occurs rarely in *Gentiana*, and up to date has been reported only in *G. macrophylla* (Wu et al., 2011). The hairy roots of *G. cruciata* (Momčilović et al., 1997), *G. macrophylla* (Zhang et al., 2010) and *G. punctata* (Vinterhalter et al., 1999) spontaneously regenerated shoots rather than somatic embryos, while *G. purpurea*, *G. scabra*, *G. triflora* x *G. scabra* required the use of different plant growth regulators for shoot regeneration (Suginuma and Akihama, 1995; Momčilović et al., 1997; Mishiba et al., 2006).

Although somatic embryogenesis in *G. utriculosa* occurred on PGR-free media, addition of cytokinins (KIN or BA) at low concentration promoted SE maturation and germination as well as the multiplication of obtained plantlets. In addition, activated charcoal, known as a good absorbent of inhibitory compounds excreted in the medium, is often used to stimulate somatic embryogenesis, androgenesis, micropropagation, protoplast culture, rooting, elongation, synthetic seed production, etc. (Thomas, 2008). However, in *G. utriculosa* AC seemingly displayed dual effect, stimulating SE

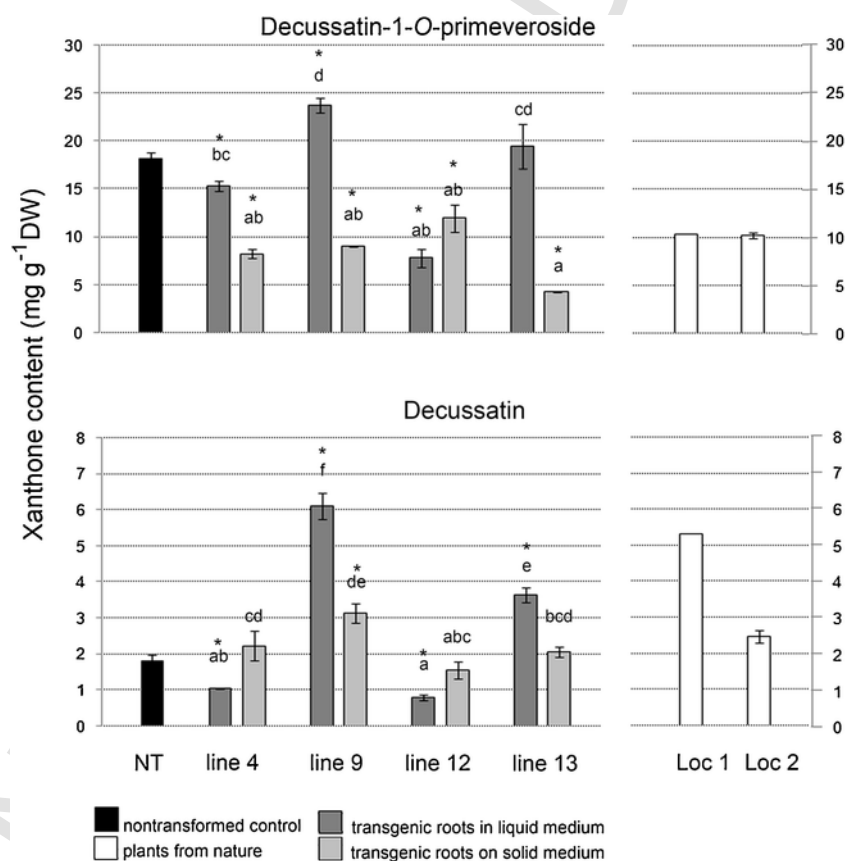


Fig. 8. Content of *Gentiana utriculosa* xanthones decussatin-1-O-primeveroside and decussatin in nontransformed control roots (NT) routinely maintained in liquid ½BM and in hairy roots of transgenic lines 4, 9, 12, and 13 grown in liquid or on solid media. Xanthone content is presented as mean values of three to five biological replicates (n = 3–5). Values marked with the same letter are not significantly different according to Fisher's LSD test at $P \leq 0.05$ following two-way ANOVA with transgenic line and nutrient media as factors. Student's *t*-test was performed to compare differences in xanthone content of each line and treatment versus NT with asterisk (*) denoting significantly different treatments at $P \leq 0.05$. Content of decussatin-1-O-primeveroside and decussatin in plant roots collected from the nature from two different localities (Loc 1 - Stara planina and Loc 2 - Divčibare) is presented in addition.

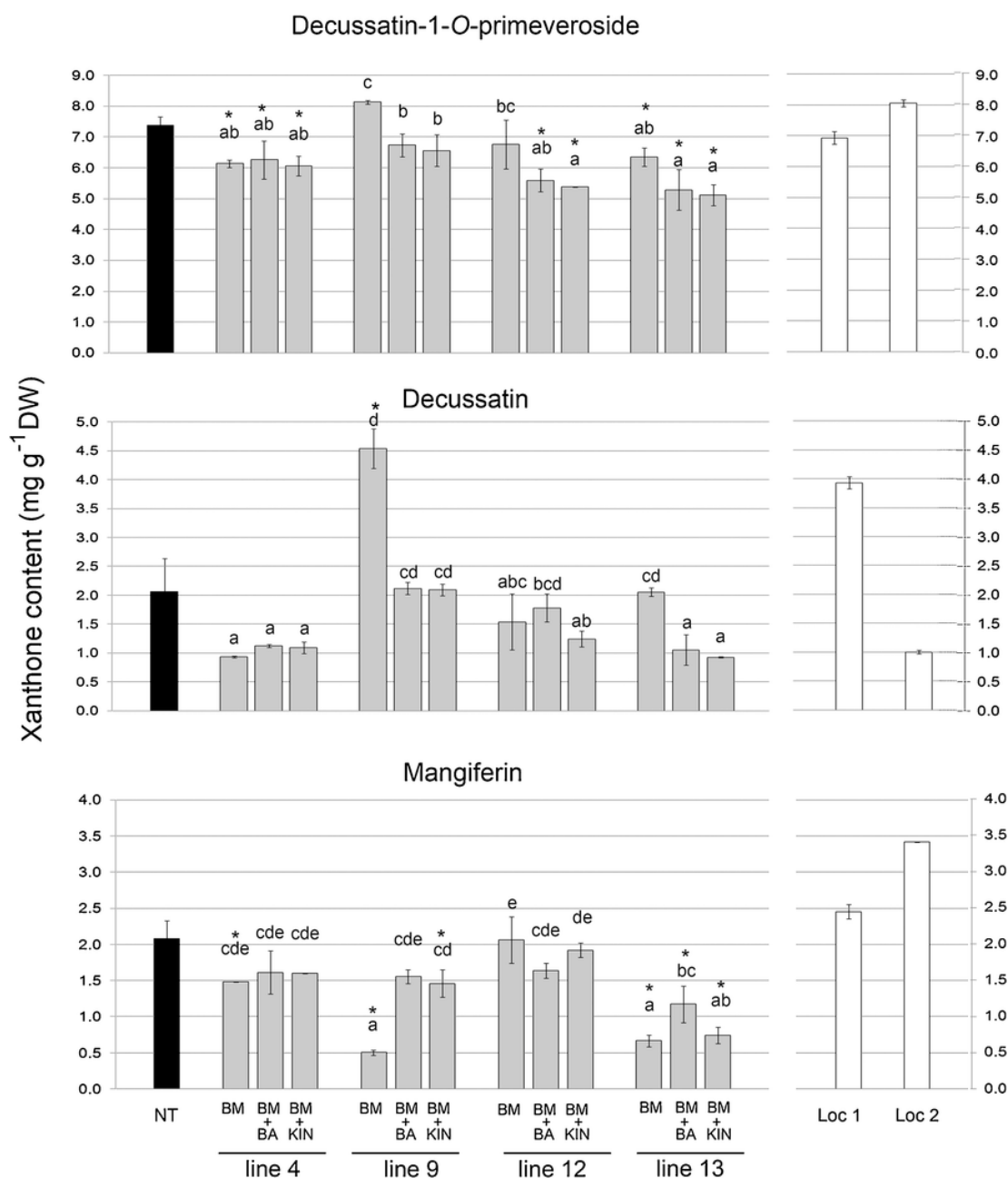


Fig. 9. Content of *Gentiana utriculosa* xanthenes decussatin-1-O-primeveroside, decussatin and mangiferin in control nontransformed shoots (NT) routinely maintained on BM supplemented with 0.2 mg L^{-1} BA and in shoots regenerated from hairy roots lines 4, 9, 12, and 13 grown on solid PGR-free BM media, or BM media supplemented with either 0.2 mg L^{-1} BA or 0.1 mg L^{-1} Kin. Xanthone content is presented as mean values of three to five biological replicates ($n = 3-5$). Values marked with the same letter are not significantly different according to the Fisher's LSD test at $P \leq 0.05$ following two-way ANOVA with the influence of transgenic line and nutrient media. Student's *t*-test was performed to compare differences in xanthone content of each line and treatment versus NT with asterisk (*) denoting significantly different treatments at $P \leq 0.05$. Content of decussatin-1-O-primeveroside and decussatin in plant shoots collected from the nature from two different localities (Loc 1- Stara planina and Loc 2- Divčibare) were presented in addition.

germination and growth and diminishing hyperhydricity of obtained plants on one hand, and inhibiting spontaneous SE induction on the other hand.

The plants regenerated from hairy roots are often phenotypically different from their mother plants, commonly having wrinkled leaves, increased branching and rooting, shortened internodes, reduced apical dominance, and altered flowering (Tepfer, 1984, 1990). Phenotypic dissimilarity was observed among transgenic *G. utriculosa* plant lines but also within the same line, and it varied from short-rosette form (most of the line 13 plantlets) to nicely

elongated (most of the line 9 plantlets) and flowering shoots (most of the line 4 plantlets).

More importantly, some hairy root-derived plants can produce higher amounts of secondary metabolites. The content of gentiopicoside in *G. macrophylla* plants, obtained through somatic embryogenesis from hairy roots, rose by 72.4% compared with wild type (WT) plants (Wu et al., 2011). Similarly, the hairy root-derived plants of *Plumbago indica* had increased plumbagin content relative to non-transformed plants (Gangopadhyay et al., 2011; Martin et al., 2011), those regenerated from *Tylophora indica* accumulated 20–60% higher augmented tylophorine content (Chaudhuri et al.,

Table 3

Two-way ANOVA tables for the effect of nutrient media type, genotype and their combination on xanthone content in lines 4, 9 and 12 of in vitro grown shoots of transgenic *Gentiana utriculosa*.

	df	Mean square	F	P
<i>Decussatin-1-O-prim</i>				
type of medium	2	2.2	4.669	0.0242
genotype	3	4.144	8.796	0.0009
type of medium x genotype	6	0.339	0.72	0.6389
<i>Decussatin</i>				
type of medium	2	2.0574	22.7433	0.0000
genotype	3	5.1034	56.4156	0.0000
type of medium x genotype	6	1.0535	11.6457	0.0000
<i>Mangiferin</i>				
type of medium	2	0.3244	4.2595	0.0316
genotype	3	1.5444	20.2761	0.0000
type of medium x genotype	6	0.2594	3.4061	0.0215

2006), while total tanshinone content in *Salvia miltiorrhiza* hairy root-derived plants was 1.8-fold and 4.7-fold higher compared with that of WT plants (Wang et al., 2013). It has been reported that the expression of *rol* genes in hairy root-regenerated plants induced the accumulation of secondary metabolites such as gentiopicoside (Wu et al., 2011) and tylophorine (Chaudhuri et al., 2006). The observed changes in phenotype and secondary metabolite production/accumulation profile might be due to position effect of T-DNA and transgene stability within plant genome, as well as the effect of Rol proteins on signal transduction and regulation of gene expression within the plant cell (Bulgakov, 2008, 2016; Dubrovina et al., 2009; Taneja et al., 2010; Mauro et al., 2017). Although the TL-DNA copy number in *G. utriculosa* clones varied from two to five, decussatin high-yielding plants contained two copies only, indicating that the higher number of T-DNA inserts does not necessarily lead to an enhanced potential for secondary metabolite production. No correlation between TL-DNA copy numbers and artemisinin content was found in *Artemisia annua* either, where hairy root line A1 with single T-DNA insert was shown to have higher growth rate and artemisinin content compared with clones that contained multiple copies (Ahlawat et al., 2014).

G. utriculosa decussatin high-yielding plants were regenerated from superior hairy root line 9, and produced the highest content of this compound. Plants of this line were also characterized by the production of decussatin-1-*O*-primeveroside at the similar level to the plants from nature, as well as of mangiferin, which was previously not detected in the hairy roots of the same line, at significantly lower level. Although the use of cytokinins BA and KIN partly enhanced mangiferin production, they were not favorable for the accumulation of two other xanthones, decussatin and decussatin-1-*O*-primeveroside. Since *A. rhizogenes*-produced transformants are mitotically stable (Lacroix et al., 2006), selected plants regenerated from *G. utriculosa* hairy roots line 9 may develop into a new cultivar for large scale production of xanthones decussatin and decussatin-1-*O*-primeveroside.

For future work, selected *G. utriculosa* hairy root culture could be subjected to enzymatic activity of different biological systems (Jiao et al., 2018) that can lead to hydrolysis of decussatin-1-*O*-primeveroside, enabling enhanced decussatin production. Hairy root culture system could also be used as a substrate for the biotransformation of decussatin into the molecules with enhanced pharmacological properties, such as its derivatives with P-glycoprotein modulator activity (Noungoue Tchamo et al., 2001). Selected elite hairy root lines can be encapsulated using synthetic seed technology and stored for prolonged time while conserving

high regeneration potential and secondary metabolite production (Gangopadhyay et al., 2011), which would facilitate their industrial use.

4.1. Conclusions

In conclusion, hairy roots as a valuable tool for sustainable production of xanthones were established in *G. utriculosa* using *A. rhizogenes* A4M70GUS strain. One of the five obtained clones was shown to produce and accumulate more than 4.5-fold higher amount of xanthone decussatin, in comparison to plants from the nature. Plants of *G. utriculosa* line 9, exhibiting stable growth for more than two years, can be considered as a favorable system for sustainable production of decussatin. It could also be a source for new bioactive substances production using biotransformation. Transgenic plants, spontaneously regenerated from this elite hairy root line through somatic embryogenesis, contained two TL-DNA copies as confirmed by Southern analysis, and displayed enhanced productivity of decussatin. Therefore, they could be valuable material to create xanthone high-yielding cultivars, which could be additionally remade by introduction of desirable genes or by modification of metabolic pathways using *A. rhizogenes*-mediated transformation.

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