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Secoiridoid glycosides production in hairy roots of *Gentiana pneumonanthe* L.: optimization of cytokinin concentration in the culture medium

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ABSTRACT: *Gentiana pneumonanthe* L. (fam. Gentianaceae) is a herbaceous perennial growing on wet heathlands, grasslands and hay meadows of Europe and Asia. Like other *Gentiana* species, *G. pneumonanthe* is characterized by the production of bitter secoiridoid glycosides (SGs) widely used in the pharmaceutical industry. Hairy roots (HRs) of *G. pneumonanthe*, as an alternative source of these phytochemicals, have been induced by agropine type *Agrobacterium rhizogenes* strain (A40M70GUS), and were characterized by genetic stability, sizable biomass production, and increased biosynthetic capacity compared to the native plant roots and above ground parts. Although HRs of *G. pneumonanthe* showed intensive growth without the application of phytohormones, the effect of varying concentrations of cytokinins (BAP – benzylaminopurine; KIN – kinetin) on the morphogenesis and on the content of SGs has been investigated in the present study, with the aim of optimizing *in vitro* conditions for enhanced production of targeted secondary metabolites. Morphogenetic changes, such as the formation of calli, were induced by both phytohormones. Significant reduction of HR biomass occurred only on treatments with high BAP and KIN concentrations (3 and 4 mg l^{-1}). The content of SGs, especially gentiopicrin and sweroside, was affected by both KIN and BAP. The lowest applied concentration of these phytohormones (0.1 mg l^{-1}) showed a stimulatory effect on the production of gentiopicrin and sweroside. Further increase in both cytokinins concentrations decreased the content of these SGs.

Being genetically stable and fast-growing, HR cultures of *G. pneumonanthe* could provide a constant and highly productive source of valuable secondary metabolites, and could further be implemented in large-scale bioreactors. Although many challenges still exist for the commercial implementation of this alternative production system, the results presented here demonstrate the opportunities for enhanced SGs production by the application of in low concentrations of both cytokinins and thus highlight the benefits of optimizing growth conditions.

Key words: *Gentiana pneumonanthe*, hairy roots, cytokinins, secoiridoid glycosides, secondary metabolites

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INTRODUCTION

Secoiridoid glycosides (SGs), a class of compounds belonging to monoterpenes, are described in the literature for their numerous pharmacological and biological activities such as antifungal and antibacterial (Šiler *et al.* 2010), as well as choleretic, anti-inflammatory, analgetic, antipyretic, pancreatic, gastroprotective and hepatoprotective (Singh 2008). These monoterpenes are present in various traditional medicine preparations (Botion *et al.* 2005; Naber 2013), and have a huge potential for use in the pharmaceutical industry. Members of the Gentianaceae family are a rich source of SGs, among which the most pharmacologically important are

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swertiamarin, gentiopicrin and sweroside (Szücs *et al.* 2002). As in the majority of the genus *Gentiana* members, these bitter principles are produced in significant amounts in *Gentiana pneumonanthe*, otherwise known as Marsh gentian (Jensen & Schripsema 2002; Szücs *et al.* 2002). This indicates the potential for these plants to be developed as a good source of SGs.

Hairy roots are developed by plants that have been infected with the soil pathogen *Agrobacterium rhizogenes*, a Gram-negative bacterium that belongs to the Rhizobiaceae family (Flocco & Giulietti 2007). These transformed tissues can be cultured under axenic conditions and are easily established and maintained in the laboratory. Their main characteristics are fast growth (0.1–2 g DW per litre per day), genetic and biochemical stability, and a pattern of metabolites similar to that of the original plant (Tepfer 1984; Sevón & Oksman-Caldentey 2002). Hairy root cultures are a useful platform for the production of valuable plant secondary metabolites at high levels, particularly alkaloids, terpenoids and phenolics (Giri & Narasu 2000; Kuźma *et al.* 2005; Wang & Wu 2010). The capacity for the biosynthesis of natural products in hairy root cultures often exceeds that of the mother plant (Kim *et al.* 2002). Therefore, multiple benefits of hairy roots are evident — they can be used for studying plant secondary metabolism through elucidation of the intermediates and key enzymes involved in their biosynthesis (Guo *et al.* 2013), they represent a valuable tool to study the biochemical properties and gene expression profiles of metabolic pathways (Gao *et al.* 2014), and they can also be used as an excellent alternative source of phytochemicals.

A variety of plant tissues produce trace amounts of phytohormones, which direct growth and influence secondary metabolite accumulation. Even though hairy roots do not require hormones in the growth medium, an exogenous supply can result in changes of hairy root morphology, more intensive growth, and an altered secondary metabolism (Vanhala *et al.* 1998; Bais *et al.* 2001; Weathers *et al.* 2005).

Here we report the successful transformation of *G. pneumonanthe* with *A. rhizogenes* A4M70GUS. The content of SGs in hairy roots was evaluated, as well as the effect of cytokinin (BAP and KIN) treatment on the morphology and secondary metabolite accumulation in transformed root tissue.

MATERIALS AND METHODS

Plant Material. Seeds of *G. pneumonanthe* were collected in 2006 in the area of Vlasina Lake (SE Serbia), and stored at -20°C until use. After 10 min surface sterilization in a 20% solution of commercial bleach with two drops of liquid detergent, seeds were rinsed 5 times with sterile distilled water. Seeds were germinated in 9 cm Petri dishes containing 20 ml solid half-strength Woody plant (½WPM) medium (Lloyd & McCown 1980), supplemented with 20 g l^{-1} sucrose, 7 g l^{-1} agar (Torlak, Belgrade, Serbia), and 100 mg l^{-1} *myo*-inositol (Merck, Darmstadt, Germany). All the cultures were grown in a growth chamber under long day conditions (16/8 h light/dark cycle), at 25 ± 2°C. White fluorescent tubes provided a photon flux rate of 32.5 µmol m^{-2} s^{-1} at the level of plant cultures.

Bacterial strain. In this study, *A. rhizogenes* strain A4M70GUS was used. This bacterial strain harbours a cointegrative plasmid with GUS construct integrated into the TL region of pRiA4 plasmid. The GUS construct contains the *uidA* sequence under the 70S promoter (enhancer-doubled 35S CaMV promoter), which is followed by the NOS polyadenylation sequence. The bacterial strain was maintained on YEB nutrient medium solidified with agar (1.5%) and supplemented with 100 mg l^{-1} neomycin (Galenika, Belgrade, Serbia). Prior to inoculation, bacterial suspensions were incubated with shaking for 24 h, at 28°C and 220 rpm.

Hairy root culture induction and maintenance. *In vitro*-grown plants were infected with *A. rhizogenes* by inoculating the internodes of stems with a sterile, hypodermic needle, dipped into bacterial suspension. Hairy roots appeared three to four weeks after the inoculation. Apical segments of primary hairy roots were excised and sub-cultured once on solid ½WPM culture medium containing 300 mg l^{-1} tolycar (Jugoremedija, Zrenjanin, Srbija). Line HR14 was selected as a putative transformant for further study, based on its phenotypic characteristics. Root tips of potentially transformed roots (HR14) and non-transformed line (R), 10 mm long, were transferred into liquid ½WPM medium and sub-cultured once a month. The cultures were grown in 100 ml Erlenmeyer flasks, containing 50 ml aerated liquid culture medium.

Confirmation of transformation by PCR. Genomic DNA was extracted from HR14, a putative hairy root line, using a CTAB isolation method. DNA from non-transformed *G. pneumonanthe* roots and bacterial DNA were used as negative and positive controls, respectively. PCR analysis was performed using the primers GUS 392 (5'-CCCGGCAATAACATACGGCGTG-3') and GUS 22 (5'-CCTGTAGAAACCCCAACCCGTG-3'), which amplified a 366 bp fragment of the *uidA* coding region. The amplification conditions were as follows: 5 min melting at 95°C followed by 35 cycles of a 1 min melting at 95°C, a 1 min annealing at 62°C and a 2 min elongation at 72°C, and final elongation for 10 min at 72°C. PCR products were visualized by electrophoretic separation on a 1% (w/v) agarose gel in 1× TBE buffer and staining with ethidium bromide.

Cytokinin treatments. Root tips (10–15 mm long) of HR14 were transferred to solid ½WPM medium with varying concentrations (0, 0.1, 0.5, 1, 2, 3 and 4 mg l^{-1}) of

either BAP or KIN. For the cytokinin treatments, twelve weeks after the onset of experiments, plant material was harvested for further analyses. For other experiments, plant material was harvested after four weeks. Both transformed and untransformed roots were cleaned from the medium by washing in distilled water for 1 min and drying using high-absorbing paper. The material was weighed (fresh weight) and air dried in a thin layer at 30°C until constant weight (dry weight - DW). The experiment was repeated three times, with 20 explants each.

HPLC analysis. Each sample (300 mg, dried and powdered) was extracted with 10 ml methanol (AppliChem, Cheshire, CT, USA) overnight. Samples were filtered through cellulose filters with 0.2 μm pore size (Agilent Technologies, Santa Clara, CA, USA) and stored at 4°C until use. Identification and quantification of SGs were performed on a HPLC-DAD system (HP1100, Hewlett Packard, Santa Clara, CA, USA). The column used for SG analyses was Hypersil BDS-C18 (Phenomonex, Torrance, CA, USA) 125 mm × 2 mm I.D. with 5 μm particle size. The mobile phase consisted of acetonitrile (HPLC grade, J.T. Baker, Deventer, The Netherlands) and 0.2% phosphoric acid. Acetonitrile (A) and phosphoric acid (B) were eluted as previously described by Mišić *et al*. (2013). The flow rate was set to 0.5 ml min^{-1} and the detection wavelength to 260 nm and 320 nm. Analyses were performed at 25°C. An additional peak confirmation was made by peak spectral evaluation with an Agilent ChemStation for LC 3D systems (Agilent Technologies, Wilmington, DE, USA), also used for the data acquisition and method/run control. Standard solutions were prepared by dissolving 10 mg gentiopicrin (>90% purity, Roth, Karlsruhe, Germany), swertiamarin, or sweroside (both 98% purity, Oskar Tropitzsch, Marktredwitz, Germany) in 10 ml methanol. Further calibration levels were prepared by diluting the stock with methanol.

Statistical analysis. Statistical analyses were performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, MD, USA). The data were subjected to Student t-test and analysis of variance (ANOVA), and treatment means were compared by the least significant difference (LSD) test calculated at the $p \leq 0.05$ confidence level. Correlation coefficients (R) and coefficients of determination (R^2) were calculated using Microsoft Excel 2000, $p < 0.001$.

RESULTS AND DISCUSSION

Hairy roots were formed at the inoculation site of stems 3 to 4 weeks after infection. The efficiency of transformation, assessed as the number of shoots developing roots per total number of inoculated explants was 10%. From 21 clonal lines of potentially transformed roots, only four were characterized by intensive growth, high degree of lateral branching, profusion of root hairs and absence of geotropism, which are the main characteristics of transformed roots. *Agrobacterium*-free hairy roots of those four lines, selected after three successive sub-cultures on tolycar and subsequently without it, grew vigorously on phytohormone-free medium. PCR amplification was used to confirm the presence of the *uidA* gene for β-glucuronidase in clonal line HR14, which was characterized by the fastest growth and most intense lateral branching. The fragment of 366 bp was successfully amplified, which strongly suggested the incorporation of the T-DNA region (Figure 1).

Hairy roots have previously been obtained from several species of the Gentianaceae family. Their production was reported for species of the genus *Centaurium* (Subotić *et al*. 2004; Piatczak *et al*. 2006; Mišić *et al*. 2013), in *Swertia japonica* (Ishimaru *et al*. 1990) and in *Blackstonia perfoliata* (Sabovljević *et al*. 2006). Most importantly, there are reports on the successful transformation of a number of gentian species with *A. rhizogenes* (Hosokawa *et al*. 1997; Momčilović *et al*. 1997; Menković *et al*. 2000; Tiwari *et al*. 2007; Hayta *et al*. 2011; Huang *et al*. 2014).

Hairy roots generally exhibit a biosynthetic capacity for secondary metabolite production, which is often greater than in their mother plants (Kim *et al*. 2002). Due to extensive branching, their growth rate is as fast as, or faster than untransformed roots. They exhibit genetic and biochemical stability which provides the stable production of secondary metabolites (Aird *et al*. 1988).

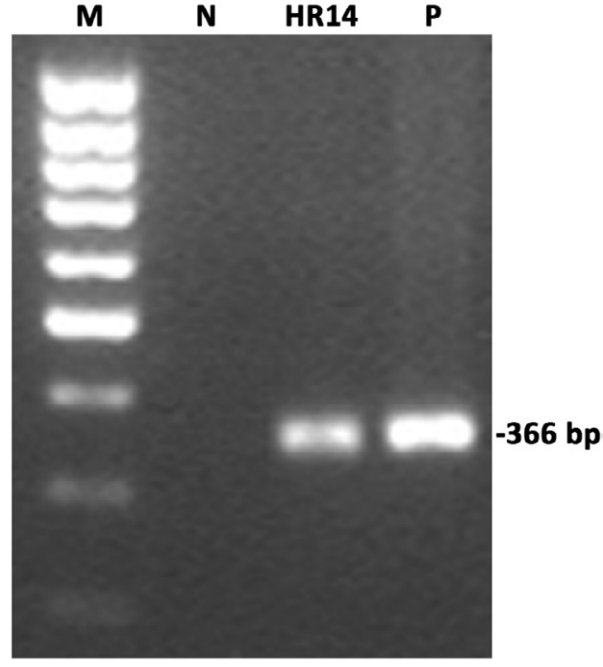

Figure 1: PCR analysis for the presence of *uidA* gene in the HR14 root lines. M - Marker (GeneRuler 100 bp DNA Ladder, Thermo Fisher Scientific, Waltham, MA, USA); P - *A. rhizogenes* A4M70GUS positive control; N - negative control; HR14 - root line containing *uidA* gene induced by *A. rhizogenes* A4M70GUS strain

The content of SGs swertiamarin, gentiopicrin and sweroside in line HR14 was determined by HPLC-DAD analysis (Figure 2). For comparison, SG contents were also evaluated in roots and aerial parts of the "mother" plant from which HR14 originated. HPLC analysis showed a more than three-fold higher content of gentiopicrin in HR14 than in untransformed roots. Contrary to gentiopicrin, sweroside content was more than 8-fold higher in untransformed roots. Similarly, in hairy root cultures of *B. perfoliata*, only trace amounts of sweroside were present, while in untransformed roots this compound was considerably more abundant (Sabovljević *et al.* 2006). Additionally, in *S. japonica*, some xanthones which were present in untransformed plants were not detected in hairy root cultures (Ishimaru *et al.* 1990). Swertiamarin production in roots didn't seem to be influenced by the process of transformation.

Although secondary metabolite biosynthesis in hairy roots is genetically controlled, various nutritional and environmental factors also have a significant influence (Toivonen 1993; Wang & Tan 2002; Ramakrishna & Ravishankar 2011). Additionally, numerous elicitors (Radman *et al.* 2003; Cheng *et al.* 2013; Ma *et al.* 2015), as well as exogenous supply of phytohormones (Yang *et al.* 2010; Gangopadhyay *et al.* 2011), have been shown to influence the yield of secondary metabolites from hairy roots. Therefore, in an attempt to increase the production of secondary metabolites, HR14 was transferred to solid ½ WPM containing either BAP or KIN at concentrations from 0.1 mg l^{-1} to 4 mg l^{-1}. Treatments with both plant growth regulators generated significant morphogenetic changes in the transformed roots. Even the lowest concentration of cytokinins induced callus formation, which was progressively more prominent at higher concentrations. Inhibition of root growth and lateral branching was also observed. While the addition of cytokinins inhibited root branching and growth even at low concentrations, the reduction in root mass was evident only at BAP concentrations higher than 2 mg l^{-1}. For KIN, significant reduction in DW was detected only at the highest applied concentration (4 mg l^{-1}) (Figure 3). Cytokinins are known to induce callus formation and to inhibit root formation at high concentrations (Robbins *et al.* 1996; Jeong *et al.* 2007; Mitić *et al.* 2012), which was also evident in the present study.

The observed external phenotypic changes did not correspond with measured secondary metabolite contents (Figure 4). The lowest concentration of cytokinins (0.1 mg l^{-1}) induced the highest production of SGs. On the other hand, concentrations above 1 mg l^{-1} were proven to be inhibitory. Decrease in the concentration of SGs in hairy roots of *G. pneumonanthe* with increasing BAP and KIN concentrations might be an indirect consequence of morphological changes induced by these phytohormones, and of the prevalence of non-differentiated tissue (calli) over highly differentiated root tissue. Non-differentiated

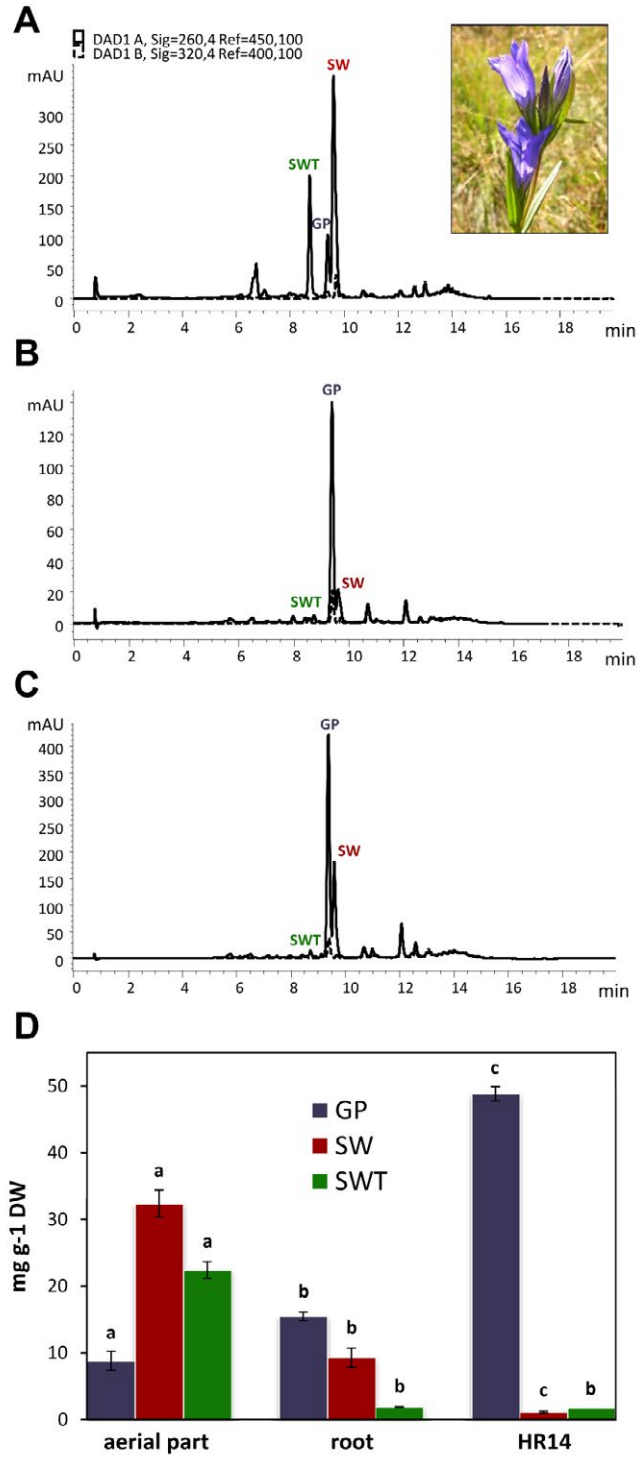

Figure 2: HPLC-DAD analyses for the identification and quantification of SGs: gentiopicrin (GP), sweroside (SW), and swertiamarin (SWT). Methanol extract profile of untransformed *Gentiana pneumonanthe* aerial parts (A), roots (B), and hairy root line HR14 (C). Secoiridoid glycosides (SGs) concentration in the analysed samples (D). Chromatograms are presented at λ = 260 nm and λ = 320 nm. Values are Means ± se of three replicates. Within each parameter, means with the same letter are not significantly different at the p≤ 0.05 level according to the Student t-test.

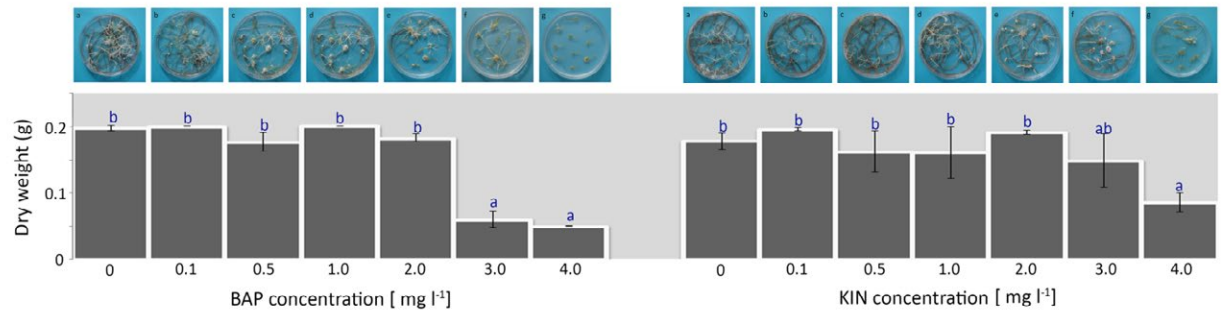

Figure 3: The effect of varying concentrations of cytokinins (KIN, BAP) on the growth of the HR14 root culture. Above each bar, a photograph of a corresponding hairy root culture is shown. Values are Means ± se of three replicates. Within each parameter, means with the same letter are not significantly different at the p≤ 0.05 level according to the Student t-test.

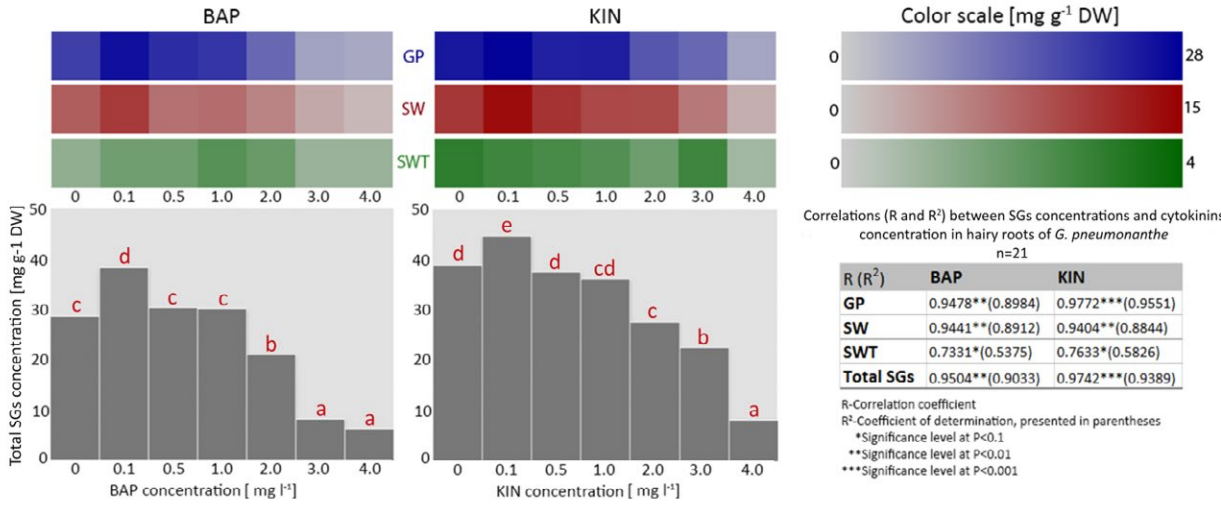

Figure 4: The effect of varying concentrations of cytokinins on the content of secoiridoid glycosides in the HR14 root culture. Heat map (upper row) based on the targeted compound content (mg g^{-1} DW) in methanol extracts of *Gentiana pneumonanthe* L. hairy roots grown on solid ½WPM media, supplemented with different BAP and KIN concentrations (0–4 mg l^{-1}). Values, which are the means of three replicates, are represented by the intensity of blue (GP), red (SW) and green (SWT) colour, as indicated in the colour scale. The content of total SGs, as influenced by BAP and KIN concentrations are represented in histograms (lower row). Means labelled with the same letter are not significantly different at the p≤0.05 level according to the LSD test. Linear regression and correlation analyses of GP, SW, and SWT contents, as influenced by BAP and KIN are presented in the Table. Abbreviations: GP – gentiopicrin; SW – sweroside; SWT – swertiamarin; SGs – secoiridoid glycosides; BAP – benzylaminopurine; KIN – kinetin.

tissues, such as calli, only rarely keep the biochemical potential of the mother plant (Łuczkiewicz *et al.* 2002; Grzegorczyk *et al.* 2007). More importantly, calli of *Gentiana dinarica*, closely related to Marsh gentian, have been shown to contain secoiridoids, but at much lower concentrations than in shoots (Vinterhalter *et al.* 2013). In members of another genus of the Gentianaceae family, *Swertia*, secoiridoids were completely absent from the callus tissues (Miura 1991).

No formation of adventitious shoots from the transformed root culture occurred, either in the absence of growth regulators or with cytokinin treatment. Cytokinins are ordinarily used as inducers of regeneration, alone or in combination with auxins (Saito & Mizukami 2002). There have been cases, though, where the use of these plant growth regulators has also resulted in significant changes in the root growth and in the accumulation of secondary metabolites (Rhodes *et al.* 1994; Farkya & Bisaria 2008). The effect of cytokinins is usually highly concentration-dependent. The effect can range from strong inhibition to significant stimulation of secondary metabolite production, depending on the amount of phytohormone that was applied. Robbins et al. (1996) have investigated the effect of different phytohormones on growth, morphology and tannin accumulation in transformed roots of *Lotus corniculatus*. They showed that BAP had little effect at low concentrations (10^{-7} M and below), but resulted in an increase in tannin levels at 10^{-6} M, which is very similar to our findings. In hairy roots of *Panax ginseng*, the application of BAP and KIN increased biomass formation

and biosynthesis of phenolic compounds (Jeong *et al.* 2007). Similar effects have been recorded in *Hyoscyamus muticus*, *Salvia miltiorrhiza* and *Polygonum multiflorum* (Vanhala *et al.* 1998; Yu *et al.* 2006; Gupta *et al.* 2011). To evaluate more precisely the influence of BAP and KIN on GP, SW, and SWT concentration in *G. pneumonanthe* hairy roots, we performed linear regression and correlation analyses (Figure 4). In the case of BAP, R-values for GP and SW were highly correlated at the $p< 0.01$ significance level, and for SWT at $p< 0.1$ level, indicating that contents of the targeted compounds were significantly influenced by BAP, and correlated with the BAP concentration. An especially strong correlation was observed for KIN and GP ($R= 0.9772$, $p< 0.001$), while SW, and especially SWT content, was less, although significantly, correlated with KIN concentration. The content of SGs was correlated with BAP ($p< 0.01$) and KIN ($p< 0.001$) concentrations, showing that KIN had a stronger influence on SG concentrations in hairy roots of *G. pneumonanthe*.

CONCLUSIONS

Here we present, to our knowledge, the first report on the production of *G. pneumonanthe* hairy roots, determination of their secondary metabolite contents, and a study on the effect of exogenous supply of phytohormones on the accumulation of SGs. The content of gentiopicrin, which is the major secoiridoid glycoside in underground parts of *G. pneumonanthe*, was higher in transformed roots, while the amount of sweroside was higher in untransformed roots. There was no change in the accumulation of swertiamarin in response to the transformation procedure. Treatment with BAP and KIN at 0.1 mg l^{-1} caused an additional increase in the amount of SGs. Hairy roots of *G. pneumonanthe* can therefore be used as a good alternative for the production of these iridoid monoterpenes, and could even be enhanced by using plant growth regulators.

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REFERENCES

Aird ELH, Hamill JD & Rhodes MJC. 1988. Cytogenetic analysis of hairy root cultures from a number of plant species transformed by *Agrobacterium rhizogenes*. *Plant Cell. Tissue Organ Cult.* **15**: 47–57.

Bais HP, Sudha G, George J & Ravishankar GA. 2001. Influence of exogenous hormones on growth and secondary metabolite production in hairy root cultures of *Cichorium intybus* L.Cv. Lucknow local. *Vitr. Cell Dev. Biol.* **37**: 293–299.

Botion LM, Ferreira AVM, Côrtes SF, Lemos VS & Braga FC. 2005. Effects of the Brazilian phytopharmaceutical product Ierobina® on lipid metabolism and intestinal tonus. *J. Ethnopharmacol.* **102**: 137–142.

Cheng Q, He Y, Li G, Liu Y, Gao W & Huang L. 2013. Effects of combined elicitors on tanshinone metabolic profiling and SmCPS expression in *Salvia miltiorrhiza* hairy root cultures. *Molecules* **18**: 7473–7485.

Farkya S & Bisaria VS. 2008. Exogenous hormones affecting morphology and biosynthetic potential of hairy root line (LYR2i) of *Linum album*. *J. Biosci. Bioeng.* **105**: 140–6.

Flocco C & Giulietti A. 2007. *In vitro* hairy root cultures as a tool for phytoremediation research. In: Willey N(ed.), Phytoremediation: methods and reviews, pp. 161–173. Humana Press, Totowa, New Jersey.

Gangopadhyay M, Dewanjee S, Chakraborty D & Bhattacharya S. 2011. Role of exogenous phytohormones on growth and plumbagin accumulation in *Plumbago indica* hairy roots and conservation of elite root clones via synthetic seeds. *Ind. Crops Prod.* **33**: 445–450.

Gao W, Sun H-X, Xiao H, Cui G, Hillwig ML, Jackson A, Wang X, Shen Y, Zhao N, Zhang L, Wang X-J, Peters RJ & Huang L. 2014. Combining metabolomics and transcriptomics to characterize tanshinone biosynthesis in *Salvia miltiorrhiza*. *BMC Genomics* **15**: 73.

Giri A & Narasu ML. 2000. Transgenic hairy roots. *Biotechnol. Adv.* **18**: 1–22.

Grzegorczyk I, Matkowski a. & Wysokińska H. 2007. Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L. *Food Chem.* **104**: 536–541.

Guo J, Zhou YJ, Hillwig ML, Shen Y, Yang L, Wang Y, Zhang X, Liu W, Peters RJ, Chen X, Zhao ZK & Huang L. 2013. CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. *Proc. Natl. Acad. Sci. U. S. A.* **110**: 12108–13.

Gupta SK, Liu RB, Liaw SY, Chan HS & Tsay HS. 2011. Enhanced tanshinone production in hairy roots of "*Salvia miltiorrhiza* Bunge" under the influence of plant growth regulators in liquid culture. *Bot. Stud.* **52**: 435–443.

Hayta S, Gurel A, Akgun IH, Altan F, Ganzera M, Tanyolac B & Bedir E. 2011. Induction of *Gentiana cruciata* hairy roots and their secondary metabolites. *Biologia* **66**: 618–625.

Hosokawa K, Matsuki R, Oikawa Y & Yamamura S. 1997. Genetic transformation of gentian using wild-type *Agrobacterium rhizogenes*. *Plant Cell Tissue Organ Cult.* **51**: 137–140.

Huang SH, Vishwakarma RK, Lee TT, Chan HS & Tsay HS. 2014. Establishment of hairy root lines and analysis of iridoids and secoiridoids in the medicinal plant *Gentiana scabra*. *Bot. Stud.* **55**: 1–8.

Ishimaru K, Sudo H, Satake M, Matsunaga Y, Hasegawa Y, Takemoto S & Shimomura K. 1990. Amarogentin, amaroswerin and four xanthones from hairy root cultures of *Swertia japonica*. *Phytochemistry* **29**: 1563–1565

Jensen SR & Schripsema J. 2002. Chemotaxonomy and pharmacology of Gentianaceae. In: Struwe L & Albert VA (eds.), Gentianaceae: Systematics and Natural History, pp. 573-631, Cambridge University Press, Cambridge, UK.

Jeong GT, Woo JC & Park DH. 2007. Effect of plant growth regulators on growth and biosynthesis of phenolic compounds in genetically transformed hairy roots of *Panax ginseng* C. A. Meyer. *Biotechnol. Bioprocess Eng.* **12**: 86–91.

Kim Y, Wyslouzil BE & Weathers PJ. 2002. Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cell. Dev. Biol. - Plant* **38**: 1–10.

Kuźma Ł, Skrzypek Z & Wysokińska H. 2005. Diterpenoids and triterpenoids in hairy roots of *Salvia sclarea*. *Plant Cell Tissue Organ Cult.* **84**: 171–179.

Lloyd G & McCown B. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmialatifolia*, by use of shoot-tip culture. In: Combined Proceedings, International Plant Propagators' Society. **30**: 421–427.

Łuczkiewicz M, Zárate R, Dembińska-Migas W, Migas P & Verpoorte R. 2002. Production of pulchelin E in hairy roots, callus and suspension cultures of *Rudbeckia hirta* L. *Plant Sci.* **163**: 91–100.

Ma P, Liu J, Osbourn A, Dong J & Zongsuo L. 2015. Regulation and metabolic engineering of tanshinone biosynthesis. *RSC Adv.* **5**: 18137-18144.

Menković N, Savikin-Fodulović K, Momčilović I & Grubišić D. 2000. Quantitative determination of secoiridoid and gamma-pyrone compounds in *Gentiana lutea* cultured *in vitro*. *Planta Med.* **66**: 96–98.

Mišić D, Šiler B, Skorić M, Djurickovic MS, Nestorović Živković J, Jovanović V & Giba Z. 2013. Secoiridoid glycosides production by *Centaurium maritimum* (L.) Fritch hairy root cultures in temporary immersion bioreactor. *Process Biochem.* **48**: 1587–1591.

Mitić N, Dmitrović S, Djordjević M, Zdravković-Korać S, Nikolić R, Raspor M, Djordjević T, Maksimović V, Živković S, Krstić-Milošević D, Stanišić M & Ninković S. 2012. Use of *Chenopodium murale* L. transgenic hairy root *in vitro* culture system as a new tool for allelopathic assays. *J. Plant Physiol.* **169**: 1203–1211.

Miura H. 1991. Swertia spp.: *In vitro* culture, regeneration, and the production of secondary metabolites. In: Bajaj YPS(ed.), Biotechnology in agriculture and forestry, vol 15. Medicinal and aromatic plants III, pp. 451–463, Springer Berlin Heidelberg, Germany.

Momčilović I, Grubišić D, Kojić M & Nešković M. 1997. *Agrobacterium rhizogenes*-mediated transformation and plant regeneration of four *Gentiana* species. *Plant Cell Tissue Organ Cult.* **50**: 1–6.

Naber KG. 2013. Efficacy and safety of the phytotherapeutic drug Canephron® N in prevention and treatment of urogenital and gestational disease: Review of clinical experience in Eastern Europe and Central Asia. *Res. Reports Urol.* **5**: 39–46.

Piatczak E, Krolicka A & Wysokinska H. 2006. Genetic transformation of *Centaurium erythraea* Rafn by *Agrobacterium rhizogenes* and the production of secoiridoids. *Plant Cell Rep.* **25**: 1308–1315.

Radman R, Saez T, Bucke C & Keshavarz T. 2003. Elicitation of plants and microbial cell systems. *Biotechnol. Appl. Biochem.* **37**: 91–102.

Ramakrishna A & Ravishankar GA. 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav.* **6**: 1720–1731.

Rhodes MJC, Parr AJ, Giulietti A & Aird ELH. 1994. Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. *Plant Cell Tissue Organ Cult.* **38**: 143–151.

Robbins MP, Evans TE & Morris P. 1996. The effect of plant growth regulators on growth, morphology and condensed tannin accumulation in transformed root cultures of *Lotus corniculatus*. *Plant Cell Tissue Organ Cult.* **44**: 219–227.

Sabovljević A, Rosić N, Janković T & Grubišić D. 2006. Secoiridoid content of *Blackstonia perfoliata in vivo* and *in vitro*. *In Vitro Cell Dev. Biol. - Plant* **42**: 427–431.

Saito K & Mizukami H. 2002. Plant cell cultures as producers of secondary compounds. In: Oksman-Caldentey KM & Barz WH (eds.), Plant Biotechnology and Transgenic Plants. pp. 77–109. CRC Press, New York, USA.

Sevón N & Oksman-Caldentey K-M. 2002. *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. *Planta Med.* **68**: 859–868.

Singh A. 2008. Phytochemicals of Gentianaceae: a review of pharmacological properties. *Int. J. Pharm. Sci. Nanotechnol.* **1**: 33–36.

Subotić A, Budimir S, Grubišić D & Momčilović I. 2004. Direct regeneration of shoots from hairy root cultures of *Centaurium erythraea* inoculated with *Agrobacterium rhizogenes*. *Biol. Plant.* **47**: 617–619.

Szücs Z, Dànos B & Nyiredy S. 2002. Comparative analysis of the underground parts of *Gentiana* species by HPLC with diode-array and mass spectrometric detection. *Chromatogr. Suppl.* **56**: 19–23.

Šiler B, Mišić D, Nestorović J, Banjanac T, Glamočlija J, Soković M & Ćirić A. 2010. Antibacterial and antifungal screening of *Centaurium pulchellum* crude extracts and main secoiridoid compounds. *Nat. Prod. Commun.* **5**: 1525–1530.

Tepfer D. 1984. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. *Cell* **37**: 959–967.

Tiwari RK, Trivedi M, Guang ZC, Guo GQ & Zheng GC. 2007. Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: Growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. *Plant Cell Rep.* **26**: 199–210.

Toivonen L. 1993. Utilization of hairy root cultures for production of secondary metabolites. *Biotechnol. Prog.* **9**: 12–20.

Vanhala L, Eeva M, Lapinjoki S, Hiltunen R & Oksman-Caldentey K-M. 1998. Effect of growth regulators on transformed root cultures of *Hyoscyamus muticus*. *J. Plant Physiol.* **153**: 475–481.

Vinterhalter B, Krstić Milošević D, Janković T, Zdravković Korać S & Vinterhalter D. 2013. Quantitative determination of secoiridoid and xanthone glycosides of *Gentiana dinarica* Beck cultured *in vitro*. *Acta Physiol. Plant.* **35**: 567–574.

Wang JW & Tan RX. 2002. Artemisinin production in *Artemisia annua* hairy root cultures with improved growth by altering the nitrogen source in the medium. *Biotechnol. Lett.* **24**: 1153–1156.

Wang JW & Wu JY. 2010. Tanshinone biosynthesis in *Salvia miltiorrhiza* and production in plant tissue cultures. *Appl. Microbiol. Biotechnol.* **88**: 437–49.

Weathers PJ, Bunk G & McCoy MC. 2005. The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. *Vitr. Cell. Dev. Biol. - Plant* **41**: 47–53.

Yang YK, Lee SY, Park WT, Park N Il & Park SU. 2010. Exogenous auxins and polyamines enhance growth and rosmarinic acid production in hairy root cultures of *Nepeta cataria* L. *Plant Omics* **3**: 190–193.

Yu R-M, Ma N, Yan C-Y & Zhao Y. 2006. Effects of exogenous phytohormones on hairy root growth and biosynthesis of anthraquinones in the hairy root culture of *Polygonum multiflorum*. *Chin. J. Biotechnol.* **22**: 619–623.

REZIME

Proizvodnja sekoiridoidnih glikozida u transformisanim korenovima vrste *Gentiana pneumonanthe* L.: optimizacija koncentracije citokinina u hranjivoj podlozi

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Gentiana pneumonanthe L. (fam. Gentianaceae) je zeljasta višegodišnja biljka vlažnih staništa i livada Evrope i Azije. Kao i ostale vrste roda *Gentiana*, *G. pneumonanthe* karakteriše proizvodnja gorkih sekoiridoidnih glikozida (SG), koji su u širokoj upotrebi u farmaceutskoj industriji. Formiranje transformisanih korenova (TK) vrste *G. pneumonanthe*, potencijalnog alternativnog izvora ovih jedinjenja, indukovano je A40M70GUS sojem bakterije *Agrobacterium rhizogenes*. U odnosu na korenove i nadzemne delove netransformisane biljke, TK je odlikovala povećana sposobnost biosinteze, a takođe i genetička stabilnost i značajan prinos biomase. Iako su TK vrste *G. pneumonanthe* pokazivali intenzivan rast i bez primene fitohormona, u ovoj studiji je ispitan efekat različitih koncentracija citokinina (BAP -benzilaminopurin; KIN - kinetin) na morfogenezu i sadržaj SG sa ciljem optimizacije *in vitro* uslova za unapređenje proizvodnje sekundarnih metabolita od interesa. Oba fitohormona indukovala su morfogenetske promene, kao što je formiranje kalusa. Do značajnog smanjenja biomase TK došlo je samo na tretmanima visokim koncentracijama hormona BAP i KIN (3 and 4 mg l^{-1}). Na sadržaj SG, posebno genciopikrina i sverozida, uticali su kako BAP, tako i KIN. Najniža primenjena koncentracija ovih fitohormona (0.1 mg l^{-1}) imala je stimulatorni efekat na proizvodnju genciopikrina i sverozida. Povećanje koncentracije citokinina dovelo je do opadanja sadržaja ovih SG.

Zbog svoje genetičke stabilnosti i brzog rasta, kulture TK vrste *G. pneumonanthe* mogu predstavljati stalni i visoko produktivni izvor sekundarnih metabolita, sa mogućnošću implementacije u industrijskim bioreaktorima. Bez obzira na mnoge izazove povezane sa komercijalnom primenom ovog alternativnog proizvodnog sistema, prikazani rezultati pokazuju mogućnost unapređenja proizvodnje SG uz pomoć primene niskih koncentracija citokinina, što ukazuje na značaj optimizacije uslova gajenja TK.