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# In Vitro Multiplication of Willow Gentian (*Gentiana asclepiadea* L.) and the Production of Gentiopicrine and Mangiferin

By

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With 1 figure

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Summary

DEVIĆ M., MOMČILOVIĆ I., KRSTIĆ D., MAKSIMOVIĆ V. & KONJEVIĆ R. 2006. In vitro multiplication of willow gentian (*Gentiana asclepiadea* L.) and the production of gentiopicrine and mangiferin. – Phyton (Horn, Austria) 46 (1): 45 – 54, with 1 figure. – English with German summary.

A protocol for in vitro propagation of *Gentiana asclepiadea* was developed. The best multiplication rate was obtained with woody plant medium (WPM) supplemented with 8.9 μM BAP (6-benzylaminopurine) and 1.1 μM IAA (indole-3-acetic acid). Gibberellic acid in the presence of 8.9 μM BAP and 1.1 μM IAA stimulated shoot elongation without affecting multiplication index.

Although spontaneous rooting on hormone-free medium was observed, auxins increased the rooting ability. Treatment with IBA (indole-3-butyric acid) induced a higher number of roots, while the addition of IAA caused an increase in root length.

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The accumulation of mangiferin and gentiopicrine, in *G. asclepiadea* grown in vitro, was much lower in roots than in shoots. The accumulation of gentiopicrine and mangiferin in cultured plants was significantly enhanced in the presence of cytokinins (BAP), even above the level of plants from natural habitat.


## Zusammenfassung

Dević M., Momčilović I., Krstić D., Maksimović V. & Konjević R. 2006. In vitro Vermehrung von Schwalbenwurz-Enzian (*Gentiana asclepiadea* L.) und die Produktion von Gentiopikrin und Mangiferin. – Phyton (Horn, Austria) 46 (1): 45 – 54, 1 Abbildung. – Englisch mit deutscher Zusammenfassung.

Es wurde ein Protokoll für die in vitro Vermehrung von *Gentiana asclepiadea* entwickelt. Die beste Multiplikationsrate ergab das „Woody Plant Medium" (WPM), versetzt mit 8.9 µM BAP (6-Benzylaminopurin) und 1.1 µM IAA (Indol-3-Essigsäure). Gibberellinsäure in Anwesenheit von 8.9 µM BAP und 1.1 µM IAA erhöhte das Längenwachstum der Sprosse ohne die Multiplikationsrate zu beeinträchtigen.

Zwar wurde auch auf hormonfreiem Medium spontane Wurzelbildung beobachtet, doch förderten Auxine die Wurzelbildung. Eine Behandlung mit IBA (Indol-3-Buttersäure) erhöhte die Anzahl der gebildeten Wurzeln, während der Zusatz von IAA eine Erhöhung der Wurzellängen zur Folge hatte. Die Anreicherung von Mangiferin und Gentiopikrin war in Wurzeln von in vitro gezogenen *G. asclepiadea* viel geringer als in entsprechenden Sprossen. Hinzufügen von Cytokinin (BAP) erhöhte die Anreicherung von Mangiferin und Gentiopikrin in kulturgezogenen Pflanzen signifikant und sogar über die Werte von Pflanzen an natürlichen Standorten.


## Introduction

*Gentiana asclepiadea* (fam. *Gentianaceae*) is a perennial herbaceous plant species distributed in South and Central Europe (Jovanović-Dunjić 1973). It belongs to the genus *Gentiana* that comprises about 400 species. Most species from this genus are threatened because of the high demand and use of bitter secoiridoid glucosides present in their roots. Gentianae radix, the officinal drug, is included in many pharmacopoeia. The constituents of the drug show beneficial effects in the treatment of gastrointestinal tract diseases. However, it has been shown recently that the aerial parts of some plants from this genus contain relatively high amounts of γ-pyrone compounds and in particular xanthones. A range of xanthone compounds actions, such as hypoglycemic, antiviral, anti-tumor, antibacterial and antioxidative effects, are listed in the current literature.

Due to the rich assortment of secondary metabolites and the high demand, not only *Gentiana lutea*, the officinal species, is endangered and put under protection in most European countries, but also other species from this genus became threatened. The protection of endangered and threatened plant species can be achieved not only by passive legal measures, but also by actively seeking alternate sources of the needed secondary metabolites as well as through propagation and reintroduction of the same species. During the last several decades, in vitro methods have proven to

be one of the most promising tools (VANISREE & al. 2004), because they combine new biotechnologies with in vitro propagation and reintroduction possibilities.

The first description of in vitro propagation of *Gentiana* species was reported for *G. cruciata* and *G. purpurea* (WESOLOWSKA & al. 1985). Since then, in vitro regeneration of *G. lutea* (VIOLA & FRANZ 1989, SKRZYPCZAK & al. 1993, MOMČILOVIĆ & al. 1997), *G. pneumonanthe* (LAMPROYE & al. 1987, MIŠIĆ & al. 2001), *G. scabra* (YAMADA & al. 1991), *G. cruciata*, *G. purpurea*, *G. acaulis* and *G. punctata* (MOMČILOVIĆ & al. 1997, VINTERHALTER & VINTERHALTER 1998) *G. davidii* var. *formosana* (CHUEH & al. 2001) could be achieved. Qualitative and quantitative determination of secondary metabolites of in vitro-cultivated plants was subsequently performed (YAMADA & al. 1991, ISHIMARU & al. 1990, MENKOVIĆ & al. 2000a,b, KRSTIĆ & al. 2003, CHUEH & al. 2001).

No reports on micropropagation of *Gentiana asclepiadea*, or on the content of major bitter glucosides and γ-pyrone compounds of in vitro cultured plants are known. In this paper we describe the successful micropropagation of *G. asclepiadea* and the accumulation of mangiferin and gentiopicrine as influenced by phytohormones.

## Material and Methods

### Plant Material

Immature fruits of *G. asclepiadea* were collected in summer 1996 on the mountain Suvobor (44°09' N; 20°11' E) and used as starting material. They were surface sterilized with 0.2% NaOCl for 15 min, followed by three times washing with sterilized water and seeds were isolated. The germination was stimulated by imbibing the seeds in sterile solution of 0.2 mM gibberellic acid ($GA_3$) for 48 h. Seeds were then transferred and grown on WPM medium ("woody plant medium", LLOYD & MCCOWN 1980) with MS (MURASHIGE & SKOOG 1962) micronutrients and organic supplements. Nodal segments (1 cm approximately) with 2–3 leaf pairs developed (30 days old plantlets) were excised and transferred to WPM medium, supplemented with 8.9 μM BAP (6-benzylaminopurine) and 2.4 μM IAA (indole-3-acetic acid). Plant material from natural populations was collected on the mountain Tara (43°26' N; 19°39' E) in June and August 2001 (in the phase of flowering).

### In Vitro Culture

In vitro propagation of *G. asclepiadea* was started from single excised shoots obtained from one seedling to provide homogenous plant material. Experiments were run on WPM medium supplemented with various concentrations of BAP ranging from 1.1 μM to 17.8 μM with constant IAA concentration of 1.1 μM. Various concentrations of $GA_3$ (7.2 μM – 115.6 μM) were used in combinations with constant concentrations of BAP (8.9 μM) and IAA (1.1 μM) to investigate the effects of gibberellic acid on stem elongation and multiplication. All experiments were carried out for 30 days. There were 30 explants per treatment and every experiment was repeated

three times. Rooting was induced by incubating 15 mm long shoots at different concentrations of IAA or IBA for 21 days.

The pH of the media was adjusted to 5.8 prior to autoclaving at 114°C for 25 min. All cultures were grown in GA7 Magenta boxes (Sigma, St. Louis, MO, USA) and kept in a growth chamber room at 25 ± 2°C, under long day conditions (16h/8h light/dark cycle). Light was provided by "Tesla" Pančevo white fluorescent tubes (photon flux density 50 μmol $m^{-2}s^{-1}$).

Statistical analyses were performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA). Data were subjected to Analysis of Variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $p < 0.05$.

### Quantitative Analysis of Mangiferin and Gentiopicrine

Plant material from nature was air-dried at room temperature to constant dry weight. Material from in vitro culture was frozen and lyophilized to constant dry weight, too. Each sample (about 400 mg, dried and powdered) was extracted with methanol for 48 hours, filtered through 0.45 μm nylon filters (Spartan-3NY, S&S Biopath, USA) and stored at 4°C until use.

Mangiferin was supplied by Sigma (Deisenhofen, Germany) and gentiopicrine by Roth (Karlsruhe, Germany). All analyses were performed using Hewlett Packard HPLC system, model 1100 with DAD (diode array detector). The column used for analysis was Hypersil BDS-C18 (5μ), 125 x 2 mm I.D. Mobile phase A was acetonitrile ($CH_3CN$, HPLC grade, Acros Organic, USA) and mobile phase B 2% aqueous solution of $H_3PO_4$. Elution by gradient was preformed according to the following scheme: phase A 2%, (2 min), A 10 % (5 min), A 20 % (10 min), A 40 % (13–18 min), A 55 % (20 min), A 90 % (23 min), A 100 % (25 min.). Flow rate was set to 0.7 ml $min^{-1}$ and the detection wavelengths to 260.4 nm and 320 nm. The injected sample volume was 5 μl.

Gentiopicrine and mangiferin quantities were calculated from calibration curves. All experiments were repeated three times. The results are presented as percentage of dry weight ± standard deviations. Standard deviations were calculated using arc-sin transformed data.

## Results

In vitro culture of *Gentiana asclepiadea* was established by the germination of seeds from immature fruits. Growth of axillary buds of excised nodal segments was stimulated with 8.9 μM BAP and 2.4 μM IAA (Fig. 1a). The effect of plant growth regulators on average shoot number and their length is presented in Tables 1 and 2. Increasing BAP concentrations in the presence of 1.1 μM IAA (Table 1) stimulated the formation of well-developed shoots (Fig. 1c), while there was a significant decrease of the shoot length in the presence of the highest BAP concentration (Table 1). Shoots elongation was stimulated in the presence of $GA_3$ at concentrations ranging from 7.2 μM to 115.6 μM (Table 2). However, higher $GA_3$ concentration (57.8 μM and 115.6 μM) led to vitrification and rapid degeneration of plants (Fig. 1b).

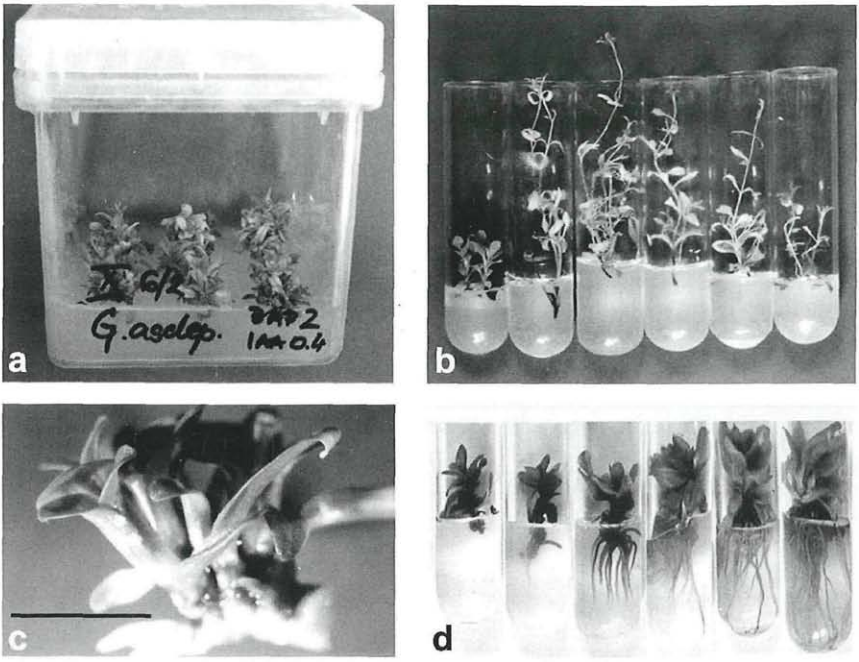


Fig. 1. In vitro multiplication of *Gentiana asclepiadea*. **a**: Established in vitro shoot culture. **b**: Effect of $GA_3$ on in vitro elongation of shoots (from left to right: control, 7.2 µM, 14.4 µM, 28.9 µM, 57.8 µM, 115.6 µM $GA_3$). **c**: Axillary buds. Bar = 5 mm. **d**: Effect of IBA on roots formation (from left to right: control, 0.5 µM, 1.2 µM, 2.5 µM, 4.9 µM, 9.8 µM IBA).

Table 1. Effect of BAP (in presence of 1.1µM IAA) on the mean number of shoots and shoot length of *Gentiana asclepiadea* explants (n = 90).

| BAP (µM) | Shoot number ± SE | Shoot length ± SE (mm) |
|---|---|---|
| 0.0 | 2.7 ± 0.3 a | 8.9 ± 0.4 a |
| 1.1 | 6.7 ± 0.3 b | 11.0 ± 0.3 ab |
| 2.2 | 7.5 ± 0.4 b | 9.8 ± 0.2 b |
| 4.5 | 6.9 ± 0.4 b | 11.3 ± 0.3 b |
| 8.9 | 7.6 ± 0.3 b | 10.4 ± 0.2 b |
| 17.8 | 8.0 ± 0.4 c | 2.1 ± 0.4 c |

Data followed by different letters are significantly different at 5% level.

Rooting was induced by different auxin concentrations with higher concentrations being more effective (Fig. 1d). After three weeks the number of roots per explant, root length and the percentage of rooting was scored (Tables 3 and 4).

Table 2. Effect of $GA_3$ (in presence of 8.9 μM BAP and 1.1 μM IAA) on mean number of shoots and shoot length of *Gentiana asclepiadea* explants (n = 90).

| $GA_3$ (μM) | Shoot number ± SE | Shoot length ± SE (mm) |
|---|---|---|
| 0.0 | 5.7 ± 0.8 ab | 8.8 ± 0.4 a |
| 7.2 | 7.8 ± 0.7 b | 18.8 ± 1.0 b |
| 14.4 | 5.6 ± 1.0 b | 21.0 ± 1.3 b |
| 28.9 | 5.2 ± 0.5 ab | 25.7 ± 1.2 b |
| 57.8 | 4.7 ± 0.8 a | 18.9 ± 1.0 b |
| 115.6 | 2.3 ± 0.9 c | 13.3 ± 0.5 c |

Data followed by different letters are significantly different at 5% level.

Table 3. Effect of IAA on rooting of *Gentiana asclepiadea* explants (n = 90).

| IAA (μM) | Mean percentage of rooted shoots ± SE | Mean number of roots per rooted shoot ± SE | Mean length of roots ± SE (mm) |
|---|---|---|---|
| 0.0 | 33.3 ± 4.9 a | 1.1 ± 0.3 a | 8.4 ± 0.6 a |
| 0.6 | 41.7 ± 5.2 ab | 1.6 ± 0.6 a | 8.4 ± 0.8 a |
| 1.4 | 27.8 ± 4.7 a | 3.3 ± 0.6 ab | 7.5 ± 0.6 a |
| 2.9 | 50.0 ± 5.3 abc | 8.9 ± 0.7 abc | 11.2 ± 0.8 ab |
| 5.7 | 63.9 ± 5.1 bc | 10.4 ± 0.5 bc | 12.3 ± 0.9 ab |
| 11.4 | 72.2 ± 4.8 c | 11.3 ± 0.5 c | 15.4 ± 0.8 b |

Data followed by different letters are significantly different at 5% level.

Table 4. Effect of IBA on rooting of *Gentiana asclepiadea* explants (n = 90).

| IBA (μM) | Mean percentage of rooted shoots ± SE | Mean number of roots per rooted shoot ± SE | Mean length of roots ± SE (mm) |
|---|---|---|---|
| 0.0 | 16.7 ± 4.0 a | 1.2 ± 0.5 a | 6.5 ± 0.6 a |
| 0.5 | 24.4 ± 4.5 a | 3.1 ± 0.8 a | 7.6 ± 0.7 ab |
| 1.2 | 35.6 ± 5.1 ab | 6.5 ± 0.6 a | 6.8 ± 0.8 a |
| 2.5 | 48.9 ± 5.3 b | 19.3 ± 0.5 b | 9.1 ± 0.8 abc |
| 4.9 | 55.6 ± 5.2 b | 20.6 ± 0.8 b | 9.7 ± 0.7 bc |
| 9.8 | 84.5 ± 3.9 c | 28.6 ± 0.7 c | 10.2 ± 0.8 c |

Data followed by different letters are significantly different at 5% level.

The results on mangiferin and gentiopicrine quantification are summarized in Table 5. The concentration of mangiferin in shoots from natural habitats is dependent on the developmental phase of the plants, because flowering shoots contain much higher amounts. In shoots from in vitro culture, mangiferin concentrations depended not only on the type of the phytohormone applied, but also on its concentration (Table 5). Only shoots grown in the presence of BAP showed satisfactory accumulation of mangiferin comparable to that of plants collected from the natural habitat in flowering phase. Both, the roots from the natural site and those obtained

from in vitro culture contained no detectable or only trace amounts of mangiferin.

Table 5. HPLC analysis of mangiferin and gentiopicrine in roots and shoots of *Gentiana asclepiadea* from in vitro culture and from natural habitats (mean values of n = 3 replicates).

| Shoots | | | | | | |
|---|---|---|---|---|---|---|
| Source of plant material | In vitro culture | | | | Natural habitat | |
| | 4.5 µM BAP | 29 µM $GA_3$ | 1.4 µM IAA | 4.9 µM IBA | Non flowering stage | Flowering stage |
| Gentiopicrine (% of dry weight ± s.d.) | 5.44 ± 0.24 | 1.18 ± 0.04 | 2.43 ± 0.09 | 0.95 ± 0.07 | 1.15 ± 0.07 | 0.66 ± 0.05 |
| Mangiferin (% of dry weight ± s.d.) | 1.77 ± 0.13 | 0.21 ± 0.02 | 0.41 ± 0.02 | 0.51 ± 0.05 | 0.18 ± 0.03 | 1.85 ± 0.11 |

| Roots | | | | |
|---|---|---|---|---|
| Source of plant material | In vitro culture | | Natural habitat | |
| | 2.9 µM IAA | 1.2 µM IBA | Non flowering stage | Flowering stage |
| Gentiopicrine (% of dry weight ± s.d.) | 0.08 ± 0.01 | 0.51 ± 0.04 | 1.83 ± 0.09 | 1.91 ± 0.10 |
| Mangiferin (% of dry weight ± s.d.) | n.d. | n.d. | n.d. | t. |

n.d. – not detected, t. – trace

The content of mangiferin in shoots grown on hormone-free medium was 0.40 ± 0.03% of dry weight while that of gentiopicrine was 1.39 ± 0.06% of dry weight.

However, the main secoiridoid compound – gentiopicrine – was detected in every plant tissue. No significant differences in the gentiopicrine concentrations between in vitro shoots and non flowering shoots harvested from natural habitats was observed. Much lower concentrations of gentiopicrine were found in flowering shoots compared to in vitro shoots. The concentration of gentiopicrine in in vitro shoots was higher than that in plants growing at natural sites irrespective of their developmental stage. However, the most effective growth regulator was BAP, which led to the highest accumulation of gentiopicrine.

## Discussion

The results show that in vitro multiplication of *G. asclepiadea* was successfully achieved via microcutting propagation. High BAP concentration increased the shoot multiplication rate. This has been already shown

in plant cultures of other *Gentiana* species (MOMČILOVIĆ & al. 1997, VINTERHALTER & VINTERHALTER 1998, CHUEH & al. 2001). Average number of shoots per explant ranged from 6 to 8. The best results with regard to shoot number and shoot length were obtained by the addition of 8.9 μM BAP in the presence of 1.1 μM IAA. Similar results with respect to optimal BAP concentrations were achieved with *G. lutea* and *G. cruciata* (MOMČILOVIĆ & al. 1997). However, optimal BAP concentration for *G. punctata* and *G. purpurea* multiplication was several times lower than that used in the present paper. In general, shoots were small and growing in rosettes. Upon application of $GA_3$ in the presence of 8.9 μM BAP and 1.1 μM IAA the shoot length increased significantly. No increase in multiplication rate was observed after addition of $GA_3$, although this has been reported for *Gentiana scabra* (YAMADA & al. 1991).

The formation of adventitious roots was frequently observed in the absence of growth regulators. Spontaneous rooting on hormone-free medium was also observed in in vitro culture of some other *Gentiana* species (MOMČILOVIĆ & al. 1997, CHUEH & al. 2001). Nevertheless, the application of auxins further improved the percent of rooting. Treatment with IBA induced higher number of roots in comparison with IAA, while addition of IAA caused a more significant increase in root length (Table 3 and 4).

The main secondary metabolites in *Gentiana* species are secoiridoids. According to the literature, the concentrations of these compounds greatly vary in *Gentiana* species (ŠAVIKIN-FODULOVIĆ & al. 2002). Results from the chemical analysis of *G. asclepiadea* growing at natural sites were published by several authors (ŠAVIKIN-FODULOVIĆ & al. 2002, KITANOV & al. 1991). They showed that the main secoiridoid in *G. asclepiadea* is gentiopicrine, which is found both in aerial parts as well as in roots (ŠAVIKIN-FODULOVIĆ & al. 2002). Our results show that the content of gentiopicrine in the aerial parts of plants before flowering, and in the roots from all developmental stages is similar. In *G. punctata* the quantity of gentiopirine was much higher in roots than in the aerial parts (MENKOVIĆ & al. 1998). The amount of gentiopicrine in roots of wild grown plants at various stages of development was also constant. However, its concentration varied in aerial parts depending on the developmental stage. The accumulation of secoiridoids in aerial parts of *G. lutea* showed a constant increase during the vegetative period (MENKOVIĆ & al. 2000c). The concentration of mangiferin in the aerial parts of *G. asclepiadea* grown in natural habitats is low and constant at the vegetative stage. However, the amount increases strongly in aerial parts of flowering plants (Table 5). The similar result was found previously for *G. lutea*. Namely, leaves of flowering plants are rich in compounds possessing C-glucoside structures while compounds with O-glucoside structures accumulate mainly before flowering (ŠAVIKIN-FODULOVIĆ & al. 2002). No detectable amounts of mangiferin were found in the

roots of wild grown *G. asclepiadea*. This is in accordance with results from KITANOV & al. 1991. Also in vitro roots contain almost no mangiferin. With regard to gentiopicrine, roots from plants cultured in vitro contain very low amounts of this secoiridoid. Generally, the accumulation of mangiferin and gentiopicrine, in *G. asclepiadea*, was much lower in roots then in shoots of plants obtained in vitro. Similar results have also been reported for other *Gentiana* species (MENKOVIĆ & al. 1998, 2000a). One of the most interesting findings of our study is that the addition of cytokinins (BAP) enhances the accumulation of gentiopicrine and mangiferin, even above the level found in wild grown plants. A similar BAP effect was also observed in shoots from in vitro culture of *Centaurium erythraea* (JANKOVIĆ 1998). In contrast, in *G. lutea* cultures, an increase in BAP level caused a decrease in the content of secondary metabolites (MENKOVIĆ & al. 2000a).

We conclude that we could develop an efficient protocol for the micropropagation of *Gentiana asclepiadea* based on the growth of axillary buds. In addition, the analyses of the accumulation and the partition of mangiferin and gentiopicrine between roots and aerial plant parts revealed that by modifying the composition of the medium, it is possible to obtain cultures of optimal conditions for large biomass yield and production of secondary metabolites. *G. asclepiadea* retained the possibility of producing secondary metabolites in vitro and, under certain conditions, the concentrations were even greater than in plants from natural habitats.

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