MASTERARBEIT | MASTER‘S THESIS

Titel | Title

Establishment of *Gentiana* root cultures

verfasst von | submitted by

Philipp Engleithner BSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Magister pharmaciae (Mag. pharm.)

Wien | Vienna, 2026

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 605
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Pharmazie
Betreut von | Supervisor:	Ass.-Prof. Mag. Dr. Christoph Wawrosch

Acknowledgments

First, I would like to thank Ass. Prof. Dr. Christoph Wawrosch for supervising this master's thesis. I am grateful for his theoretical and practical guidance throughout the entire process, his patience, excellent communication, and his ability to always offer a solution whenever a problem arose.

I would also like to thank Univ.-Prof. Dr. Sergey B. Zotchev for the opportunity to write this thesis in the Pharmaceutical Biotechnology Group.

Furthermore, I thank Mag. Silvia Löbsch for ensuring a smooth workflow in the lab, as well as for her helpful tips in day-to-day lab work.

Thanks also go to Dr. Olha Schneider for her crucial support with the HPLC, and to MMag. pharm. Nicole Schranzer, BA, BSc, for her help with the PCR and her advice on day-to-day lab work.

Special thanks to Berni-Mary Mappalakyil, BSc, for the valuable tips and the introduction at the start of my project.

Finally, I would like to thank my family and friends for both their financial support and for always listening to me during this time, as well as for their interest in my work and their help with proofreading.

Table of contents

1. Abstract 4
2. Introduction 6
   2.1. *Gentiana* species 6
   2.2. Plant *in vitro* culture and secondary metabolites of *Gentiana* species 8
   2.3. Hairy Roots 12
3. Aim of this work 14
4. Material and Methods 15
   4.1. Plant material 15
   4.2. Plant culture media 15
   4.3. Plant culture conditions 15
   4.4. Sterilization of seeds and pretreatments 16
   4.5. *Rhizobium rhizogenes* and growth behaviour of strain A4 17
   4.6. Detection of *rolB* gene in *Rhizobium rhizogenes* strains 17
   4.8. Infection experiments with scalpel 18
   4.9. Infection experiments through co-cultivation 19
   4.9. Adventitious root culture 20
   4.10.1. Sample preparation 21
   4.10.2. HPLC parameters 21
   4.10.3 Calibration curves of standard compounds 22
5. Results 23
   5.1. Germination of *Gentiana* species 23
   5.2. Growth of *R. rhizogenes* strain A4 27
   5.3. Detection of *rolB* gene 28
   5.4. Establishment of hairy root cultures 29
   5.5. Establishment of adventitious root cultures 31
   5.6. HPLC analysis of adventitious root cultures 33
6. Discussion 42
7. References 47

Abbreviations

BAP: 6-Benzylaminopurin

GA_3: Gibberellic acid

HPLC: High performance liquid chromatography

MS: Murashige and Skoog medium

1/2MS: half-strength Murashige and Skoog medium

NAA: 1-Naphthaleneacetic acid

OD: Optical density

PCR: Polymerase chain reaction

RPM: Revolutions per minute

SAAT: Sonication-Assisted Agrobacterium Transformation

1. Abstract

The genus *Gentiana* is of great interest to the pharmaceutical and food industries due to its secondary metabolites like e.g. the secoiridoid glycosides gentiopicroside, swertiamarin, and amarogentin. Because of the endangered status of wild populations of *Gentiana lutea* L. and the long growth period required for the roots to reach harvest maturity, this study aimed to establish hairy root cultures of *Gentiana lutea* L., *G. cruciata* L, *G. purpurea* L, and *G. asclepiadea* L. as a sustainable biotechnological alternative for metabolite production. *In vitro* seed germination protocols using ½ MS medium were established for these *Gentiana* species. Gibberillic acid (GA_3) pretreatment (500 mg· L^{-1}) combined with incubation at 25 °C resulted in a germination rate of nearly 100% for *G. cruciata*. In contrast, a lower temperature of 18 °C proved to be crucial for the successful development of *G. lutea* and *G. asclepiadea*, resulting in approximately 40% and 35% viable seedlings, respectively. 499 seedlings were inoculated with *Rhizobium rhizogenes* strain A4. Despite of using various infection methods, hairy root formation was not observed in any of the species. This suggests that strain A4 or the specific inoculation parameters used may not have been optimal for these species. For comparison in future studies, adventitious root cultures were successfully established in *G. cruciata* and *G. lutea* using 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). HPLC analysis revealed that while gentiopicroside was present in the *in vitro* adventitious root cultures, the concentration (max. 0.11% in *G. lutea*) was significantly lower than the 2.65% found in commercially available *Gentianae radix*. Swertiamarin and amarogentin were not quantifiable in the adventitious root cultures. These results suggest that the accumulation of metabolites in *Gentiana* root cultures is strongly influenced by the plant's developmental stage and environmental factors, and further studies on alternative bacterial strains and elicitation methods are required to achieve successful hairy root induction and higher metabolite yields.

Zusammenfassung

Die Gattung *Gentiana* ist aufgrund ihrer Sekundärmetabolite wie die Secoiridoidglykoside Gentiopikrosid, Swertiamarin und Amarogentin von großem Interesse für die pharmazeutische und Lebensmittelindustrie. Aufgrund des gefährdeten Status wilder Populationen von *Gentiana lutea* L. und der langen Wachstumsphase, die die Wurzeln bis zur Erntereife benötigen, zielte diese Studie darauf ab, „hairy roots“ von *G. lutea*, *Gentiana cruciata* L., *Gentiana purpurea* L. und *Gentiana asclepiadea* L. als nachhaltige biotechnologische Alternative für die Metabolitenproduktion zu etablieren. Für diese Gentiana-Arten wurden *in-vitro*-Keimungsprotokolle unter Verwendung von ½-MS-Medium etabliert. Eine Vorbehandlung mit Gibberellinsäure (GA_3; 500 $mg \cdot L^{-1}$) in Kombination mit einer Inkubation bei 25 °C führte bei *G. cruciata* zu einer Keimungsrate von fast 100 %. Im Gegensatz dazu erwies sich eine niedrigere Temperatur von 18 °C als entscheidend für die erfolgreiche Entwicklung von *G. lutea* und *G. asclepiadea*, was zu etwa 40 % bzw. 35 % lebensfähigen Sämlingen führte. 499 Sämlinge wurden mit dem *Rhizobium rhizogenes* Stamm A4 inokuliert. Trotz des Einsatzes verschiedener Infektionsmethoden konnte bei keiner der Arten die Bildung von hairy roots beobachtet werden. Dies deutet darauf hin, dass der Stamm A4 oder die verwendeten spezifischen Inokulationsparameter für diese Arten möglicherweise nicht optimal waren. Zum Vergleich für zukünftige Studien wurden bei *G. cruciata* und *G. lutea* unter Verwendung von 1-Naphthylessigsäure (NAA) und 6-Benzylaminopurin (BAP) erfolgreich Adventivwurzelkulturen etabliert. Die HPLC-Analyse ergab, dass Gentiopikrosid in den *in-vitro*-Adventivwurzelkulturen zwar vorhanden war, die Konzentration (max. 0,11 % bei *G. lutea*) jedoch signifikant niedriger war als die in kommerziell erhältlicher *Gentianae radix* gefundenen 2.65 %. Swertiamarin und Amarogentin waren in den Adventivwurzelkulturen nicht quantifizierbar. Diese Ergebnisse legen nahe, dass die Akkumulation von Metaboliten in *Gentiana* Wurzelkulturen stark vom Entwicklungsstadium der Pflanze und von Umweltfaktoren beeinflusst wird und weitere Studien über alternative Bakterienstämme und Elizitierungsmethoden erforderlich sind, um eine erfolgreiche hairy root-Induktion und höhere Metabolitenerträge zu erzielen.

2. Introduction

2.1. *Gentiana* species

The Genus *Gentiana* is a member of the family Gentianaceae. There are about 400 species worldwide, of which most can be found in temperate zones and areas of higher elevation like Himalaya, subalpine and alpine regions. Morphologically, the genus consists mainly of perennial herbaceous plants, although the wider family can include annuals, shrubs, and lianes, depending on the habitat (Mirzaee et al., 2017). Known since ancient times for their medicinal properties, plants of the genus *Gentiana* have long been the focus of both traditional folk medicine and modern pharmacological research (Pan et al., 2016). Many *Gentiana* species are also used for industrial purposes. In food industry, the development of alcoholic bitter beverages is a key focus. Parts of the plant are used in folk medicine to improve digestion and to stimulate the appetite (Xu et al., 2017). Studies have demonstrated a variety of pharmacological and biological activities including anti-inflammatory, gastroprotective and hepatoprotective (Božić et al., 2015). The European Pharmacopoeia contains two monographs, *Gentianae radix* and the tincture produced from it, *Gentianae tinctura*. *Gentiana lutea* L. is the official plant species used according to the pharmacopoeia (*Europäisches Arzneibuch*, 2023). Other species such as *Gentiana pannonica* Scop., *Gentiana punctata* L. and *Gentiana purpurea* L. used to be official in various pharmacopoeias. However, due to their smaller root mass, they no longer play a significant role (Blaschek et al., 2014). The collection of roots from wild populations of *G. lutea* threatens the species, which is why it is covered by the Habitats Directive in Austria and several other European countries. A European directive on the conservation of natural habitats and wild fauna and flora The Habitats Directive on the conservation of natural habitats and of wild fauna and flora aims to ensure the protection of biodiversity by requiring Member States to maintain or restore natural habitats and species of Community interest to a favourable conservation status (Ehrendorfer et al., 2022). Seeds undergo morphophysiological dormancy and require at least 60–90 days at temperatures near freezing to break dormancy and germinate. Due to this, global

warming also has an impact on the distribution of *Gentiana lutea* (Cuena-Lombraña et al., 2018).

The species *G. lutea* L. (figure 1, page 7), *G. cruciata* L. (figure 2, page 7),*G. asclepiadea* L. (figure 3, page 8) and *G. purpurea* L. (figure 4, page 8) are native to Austria. Depending on the author, the genus *Gentiana* is further divided into 7 to 11 sections. Gentiana is the most important section, at least in Europe, as it is of great importance as a source of drugs. The section includes *G. lutea* and *G. purpurea*, among others. *G. lutea* occupies a special position, which is also evident in chemotaxonomy. *G. asclepiadea* belongs to the section Pneumonanthe and *G. cruciata* to the section Aptera, which has been chemotaxonomically justified (Blaschek et al., 2014). Due to their slightly different phytochemistry, these species were selected for this study.

Figure 1*: Gentiana Lutea* L.

https://de.wikipedia.org/wiki/Datei:Gentiana_lutea_090705.jpg [20.03.2026]

Figure 2: *Gentiana cruciata* L.

https://de.wikipedia.org/wiki/Datei:Gentiana_cruciata_030705a.jpg [20.03.2026]

Figure 3: *Gentiana asclepiadea* L.

https://de.wikipedia.org/wiki/Datei:Gentiana_asclepiadea_Puglalm.jpg [20.03.2026]

Figure 4*: Gentiana purpurea* L.

https://de.wikipedia.org/wiki/Datei:Gentiana_purpurea_Swiss.jpg [20.03.2026]

2.2. Plant *in vitro* culture and secondary metabolites of *Gentiana* species

The therapeutic efficacy of the genus *Gentiana* is attributed to its rich range of secondary metabolites, of which over 600 compounds have been identified to date (Falcone et al., 2025). The most characteristic components are secoiridoid glucosides such as gentiopicroside (figure 5, page 9), loganic acid, sweroside, and swertiamarin (figure 6, page 9), which are responsible for the typical bitter taste of the plants and have been shown to have anti-inflammatory and liver-protective effects (Xu et al., 2017). Amarogentin (figure 7, page 9) stands out as the most bitter natural substance known, with a bitterness index of 58,000,000 compared to 200,000 achieved by quinine (Keil et al., 2000). In addition, the genus *Gentiana* is an important source of xanthones, flavonoids, triterpenoids, and monoterpene alkaloids. More than 120 highly oxygenated iridoids with different structures have been discovered in *Gentiana* species, such as secoiridoids and mixed iridoid-secoiridoid structures. Most iridoids in this genus are glycosidic in nature. Iridoids have a cyclopentanopyran ring. In the genus *Gentiana* they contain oxygen at C(1) and C(7), while C(11) is present as a COOH or ester group (Pan et al., 2016).

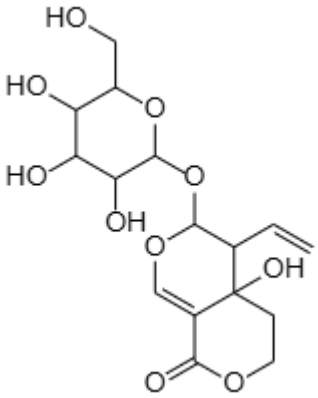


Figure 5: Chemical structure of gentiopicroside.

https://lotus.naturalproducts.net/compound/lotus_id/LTS0210938 [20.03.2026]

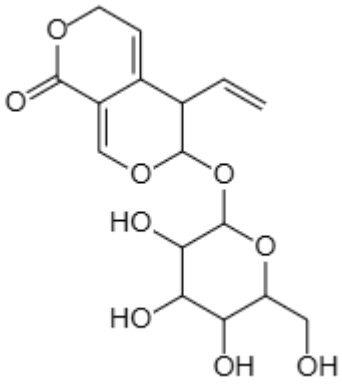


Figure 6: Chemical structure of swertiamarin.

[https://lotus.naturalproducts.net/compound/lotus_id/LTS0241296 [20.03.2026]

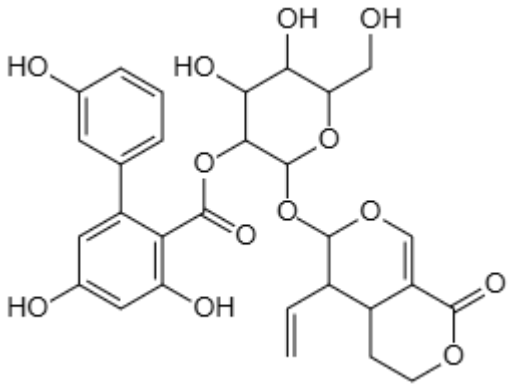


Figure 7: Chemical structure of amarogentin.

https://lotus.naturalproducts.net/compound/lotus_id/LTS0061135 [20.03.2026]

Gentiopicroside is the most common compound in *Gentiana* species (Xu et al., 2017). In vitro and in vivo studies showed that gentiopicroside displays many biological activities. Gentiopicroside exhibits anti-inflammatory activity through selective inhibition of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Antoniadi et al., 2023; Ren et al., 2023). In addition, gentiopicroside shows potential for anti-cancer activity. Li et al. (2019) demonstrated that gentiopicroside has an anti-cancer effect on SKOV3 ovarian cancer cells by inducing apoptosis and G2/M cell cycle arrest. Further research showed that gentiopicroside leads to a recovery from the lipid accumulation induced by acute and chronic alcohol intake. Targeting the P2X7 receptor signalling pathway by gentiopicroside could be a therapeutic option for treating alcoholic hepatic steatosis (Li et al., 2018).

Swertiamarin also exhibits a number of pharmacological properties. These include anti-atherosclerotic, antidiabetic, anti-inflammatory and antioxidant effects (Leong et al., 2016). In experimentally induced myocardial infarction in rats, swertiamarin showed cardioprotective effects through reduced oxidative stress and decreased inflammatory response (Wang et al., 2023). Antidiabetic effects are caused by activating ADRB3/UCP1 thermogenic signals in adipose tissue (Chen et al., 2024). Another study demonstrated the neuroprotective effect of swertiamarin in the rotenone mouse model of Parkinson's disease (Sharma et al., 2023).

Amarogentin also has antitumorigenic properties. In gastric carcinoma cells, amarogentin can decrease gastric carcinoma multiplication and induces apoptosis (Tan et al., 2022). In a sepsis-induced brain injury model in mice, amarogentin inactivates the NF-κB pathway by increasing AMPK/SIRTI levels, exerting anti-inflammatory, antioxidant stress, and anti-apoptotic neuroprotective functions (Song & Zhou, 2022).

The therapeutic versatility of the secondary metabolites of *Gentiana* has led to increased global demand, particularly for the roots of the plants, which in the past was met by the destructive harvesting of wild stocks (Vinterhalter et al., 2021). Another problem is the long growing period required for the roots to reach harvest maturity. In cultivation trials of *G. lutea*, this takes 4–6 years to produce economically acceptable yields (Radanović et al., 2014).

To strike a balance between the needs of industry (some 6,000 tons of fresh roots per year (Seitz et al., 2005)) and the necessity of preserving wildlife diversity, research has focused on sustainable production alternatives. One such strategy involves biotechnological systems such as plant tissue cultures (Marković et al., 2019).

Plant cell and tissue culture, often referred to as *in vitro* culture, involves the growth of plant cells, tissues, and organs under controlled aseptic conditions using special nutrient media (Espinosa-Leal et al., 2018). Controlled laboratory environments enable the standardization of extracts and can often be optimized to achieve metabolite concentrations that are significantly higher than those found *in vivo* (A. Salim et al., 2021).

In vitro callus formation results in dedifferentiated cell masses that can be utilized to establish cell suspension cultures in liquid nutrient media. Cell suspension cultures are often used to produce secondary metabolites due to their rapid growth and suitability for scaling up in bioreactors. One of the most prominent examples is paclitaxel (Taxol®), an anti-cancer drug from *Taxus* species (Tabata, 2004). In higher plants, however, certain biochemical properties are only expressed in specific organs (Wawrosch & Zotchev, 2021). Substances that are specifically biosynthesized in roots can be obtained from adventitious root cultures. The formation of plant roots can be induced from organs other than the root itself under suitable, hormone-supported conditions. Unlike undifferentiated cell suspensions, adventitious root cultures exhibit greater consistency in metabolite production, making them a promising method for large-scale biomass production (Rahmat & Kang, 2019). For even greater efficiency, hairy root cultures are used as a transgenic alternative (Ramachandra Rao & Ravishankar, 2002).

2.3. Hairy Roots

Hairy roots are root like tissues generated by the infection of plants with *Rhizobium rhizogenes* (formerly classified as *Agrobacterium rhizogenes*). *Rhizobium rhizogenes* is a Gram-negative soil bacterium that functions as a natural plant pathogen (Gutierrez-Valdes et al., 2020). Chilton et al. (1982) demonstrated that, like *Agrobacterium tumefaciens*, the Ri (root-inducing) plasmid in *R. rhizogenes* possesses the ability to transfer T-DNA (transferred DNA) into the plant genome. Expression of genes on the T-DNA stimulates the transformed tissue to produce large amounts of auxin, leading to excessive root growth. The transformed tissues also produce opines, compounds derived from amino acids, which supply *R. rhizogenes* with nitrogen and carbon (Zhu et al., 2024). The key genes on the T-DNA are the (rol) genes (root locus) *rolA*, *rolB*, *rolC*, and *rolD*. These regulate the plant's hormone balance. *rolA* and *rolC* influence cytokinin metabolism, *rolB* increases auxin sensitivity and *rolD* influences sugar metabolism (Liu et al., 2025).
The Ri plasmid also contains the *vir* gene cluster, which encodes most of the proteins required to mediate DNA transfer, as well as the T-DNA itself. The *vir* gene cluster is activated when injured plant tissue releases phenolic compounds such as acetosyringone (AS; 3,5-dimethoxyacetophenone), as illustrated in figure 8 (page 13). T-DNA and virulence proteins are transported via T4SS (type IV secretion system) (Lacroix & Citovsky, 2019). The final steps of this transformation mechanism involve the cytoplasmic transport and import of the T-DNA and the effector proteins into the nucleus of the plant cells, their integration into the plant genome, and the expression of the T-DNA (Hwang et al., 2017).
Hairy roots have the advantage of hormonal autotrophy, which allows them to grow without exogenous plant growth regulators. Moreover, they are characterized by exceptional genetic and biosynthetic stability, enabling them to maintain a constant level of secondary metabolite production over long periods of time (Zhu et al., 2024). The use of *in vitro* cultures and biotechnological methods such as hairy root cultures can be highlighted as a key strategy for the sustainable production of valuable secondary metabolites in the genus *Gentiana*. Momčilović et al. (1997b) have successfully transformed *G. lutea*, *G. cruciata*, and *G. purpurea* using wild-type strains such as ATCC 15834 and A4, resulting in permanent, fast-growing root

cultures. However, they did not examine the content of secondary metabolites in these *Gentiana* species. Hayta et al. (2011) described the induction of *Gentiana cruciata* hairy roots and the formation of the secondary metabolites gentiopicroside and swertiamarin. Studies on *G. asclepiadea* are less frequent in the context of hairy root transformation. A comparative study of *G. lutea*, *G. cruciata*, *G. asclepiadea*, and *G. purpurea* would be helpful, as the content of secondary metabolites is strongly influenced by the environment, the age, and the specific genotype of the plant. The analysis of these four species for their content of gentiopicroside, swertiamarin, and amarogentin is of particular interest due to the high medicinal value and chemical diversity within the genus. Such research is vital for the pharmaceutical industry to reduce its dependence on endangered natural habitats (Tiwari et al., 2007). Previous research on *G. dinarica* hairy roots suggests that different clones within the same species may exhibit significant differences in their metabolite profiles due to the positional effects of T-DNA integration (Vinterhalter et al., 2015).

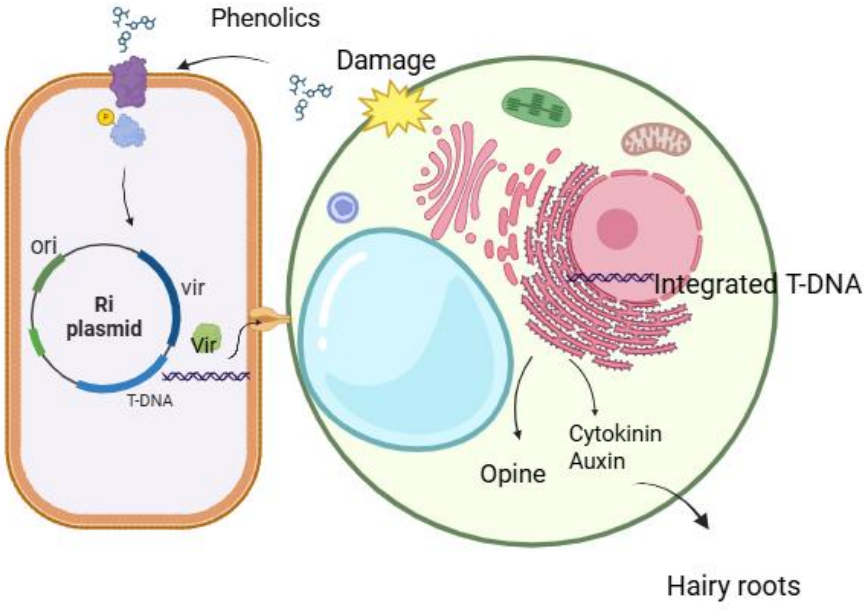


Figure 8: Diagrammatic representation of the cellular mechanism of *Rhizobium rhizogenes*–mediated transformation of the host cell.

Created in Biorender.com

3. Aim of this work

The main aim of the work was the establishment of root cultures of *G. lutea, G. cruciata, G. purpurea* and *G. asclepiadea* and quantification of the secondary metabolites gentiopicroside, swertiamarin and amarogentin. To achieve this goal, several steps had to be taken.

- *In vitro* germination of the selected species

Surface sterilization of seeds and subsequent germination and growth under sterile conditions.

- Confirmation of the bacterium's functional properties

PCR analysis to confirm the presence of the rolB gene in *Rhizobium rhizogenes.*

- Establishment of hairy root cultures

Infection of seedlings with *Rhizobium rhizogenes.*

- Establishment of adventitious root cultures

Cultures of normal roots to be established from axenically grown seedlings.

- Quantification of gentiopicroside, swertiamarin and amarogentin

Using HPLC, a calibration curve is generated and the concentration of gentiopicroside, swertiamarin and amarogentin in the roots is quantified.

4. Material and Methods

4.1. Plant material

All seeds used were purchased from Jelitto Staudensamen GmbH, Schwarmstedt, Germany. The seeds had the following batch numbers: *Gentiana purpurea* GA1668001103AA4AA0, *Gentiana cruciata* GA09870090015AA5AA0s, *Gentiana asclepiadea* GG08670373022AA1AF1s and *Gentiana lutea* GG13470093030AA3AC1s. *Gentiana asclepiadea* and *Gentiana lutea* seeds were GOLD NUGGET SEED®**.** GOLD NUGGET SEED® make it possible for cold germinators to germinate evenly within 2 to 4 weeks without cold treatment. The seeds were stored at 4°C.

4.2. Plant culture media

Three different media were used for seed germination. These were modified half-strength semisolid MS medium (1/2MS) with 30 g/L sucrose (Murashige & Skoog, 1962), Gamborg B5 (Gamborg et al., 1968) and M29 medium, a modified half strength MS medium containing 10g/L sucrose. All chemicals used for media preparation were of biochemical grade, except sucrose which was of household quality. The media were solidified with 3 g/L Gelrite® (Carl Roth GmbH + Co KG, Karlsruhe, Germany) and the pH value adjusted to 5.6-5.8 using 1 M NaOH. The medium was filled into 100 ml baby food jars (Hipp®) in 40 ml portions and sealed with Magenta™ Caps. The media were then autoclaved at 121°C for 15 minutes and stored at 18°C.

4.3. Plant culture conditions

For *in vitro* cultivation, after sterilization, a portion of the seeds underwent cold stratification at 4 °C to break dormancy. The respective experimental batches were

then incubated at either 18 °C or 25 °C under an ambient relative humidity of 50%. All cultures were kept under a 16 hours photoperiod with a light intensity of 40 $\mu M \cdot m^{-2} \cdot s^{-1}$. All 4 species were tested under different conditions to achieve successful germination. Seeds of *Gentiana purpurea* and *G. cruciata* were incubated at a temperature of 4°C for 2, 3, 4, and 5 weeks, both with and without gibberellic acid (GA_3) (Sigma Aldrich) pretreatment. For this pretreatment seeds were soaked in an aqueous solution of 500 mg L^{-1} GA_3 for 24 hours prior to surface sterilization, which can increase the germination rate of dormant seeds (González-López & Casquero, 2014). After the 4 °C period, the seeds were kept at 25°C. In another experiment seed cultures with GA_3 pretreatment were initially incubated at 25°C for 2 weeks, followed by 6 weeks at 4°C, and then again at 25°C. Germination of *G. asclepiadea* seeds was tested at 18°C and 25°C with and without GA_3, while seeds of *G. lutea* were studied at 18°C and 25°C with and without GA_3 on hormone-free Gamborg B5, 1/2 MS, and M29 medium to obtain optimal germination conditions.

4.4. Sterilization of seeds and pretreatments

50 seeds per treatment were used for the germination tests. Once the optimal germination conditions were known, this number was increased to 80. The seeds were wrapped in transparent curtain fabric cut to a size of approximately 10 x 10 cm together with 10 glass beads and secured with sewing thread so that they would not fall out. This bag was placed in a 50 ml Falcon tube and a 1:10 to 1:4 (10-25 %) dilution (1.4-3.5 % active chlorine) of a commercial concentrated NaOCl solution (W. Neuber's Enkel GmbH, Vienna, Austria; 14% active chlorine) with addition of 2 drops of Tween® 20 (Sigma Aldrich) was added. In order to keep the sterilization concentration and sterilization time as low as possible, the test series was reduced from an initial 25% and 15 minutes to 10% and 10 minutes. After disinfection the seeds were rinsed with autoclaved water and were then kept in autoclaved water for 10 minutes. The latter washing step was repeated three times. After the seeds had been blotted dry on sterile filter paper, 5-8 seeds per jar were transferred to 1/2 MS medium and the containers sealed with Parafilm®.

4.5. *Rhizobium rhizogenes* and growth behaviour of strain A4

Rhizobium rhizogenes strain A4 obtained from Dr. Szabolcs Béni (Department of Pharmacognosy, Semmelweis University, Budapest, Hungary) was used for the infection experiments. The strain was cultivated on YEB medium (yeast extract 1 g/L, beef extract 5 g/L, peptone 5 g/L, sucrose 5 g/L, MgSO4 7 H2O 0.49 g/L, agar 15 g/L, pH=7.2) and stored at 4°C in the dark.

A number of factors are known to influence the experimental induction of hairy roots, one of these being the bacterial concentration. A bacterial culture at an OD_{600} of 0.6-0.8 has been successfully used to induce hairy roots in various plant species (Zhu et al., 2024). In order to have the bacterial culture ready for use after two days we followed a protocol developed by Mappalakayil (2026). A colony of *Rhizobium rhizogenes* strain A4 was used to inoculate 2 mL of YEB, this pre-culture was incubated at 28°C and 200 rpm for 19 hours and 30 minutes. Subsequently, 800 µL of the pre-culture was transferred to 20 mL YEB medium and further cultivated at 28°C and 200 rpm. Measurements were taken at various intervals over a 5-hour period, with 1-ml samples collected and analysed at each interval.

4.6. Detection of *rolB* gene in *Rhizobium rhizogenes* strains

Before using *Rhizobium rhizogenes* for infection experiments it was checked for presence of the Ri plasmid. This was done through a PCR analysis to detect the presence of the *rolB* gene. We examined the A4 strain which was used in our study, and a newly acquired strain ATCC15834 (obtained by Prof. Alfredo Ambrosone, Department of Pharmacy, University of Salerno, Italy).

For DNA extraction, one colony each was incubated in 2 ml YEB medium and grown overnight at 28°C and 200 rpm. Afterwards, 1.5 ml of the bacterial suspension was transferred to a 1.5 ml Eppendorf tube, centrifuged and the supernatant removed. The plasmid was then isolated using the Wizard®PlusSV Minipreps DNA Purification System (Promega GmbH, Walldorf, Germany).

Primer sequences homologous to those of the *rolB* gene were used for PCR amplification. As described by Moussous et al., (2018) and Shakeran et al., (2017),

these were 5'-TGGATCCCAAATTGCTATTCACGA-3' (forward) and 5'TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3' (reverse). The primer stock solutions were reconstituted with autoclaved water according to the delivery note to a concentration of 100 pmol μL^{-1}. The volume for the stock solution was 276 µL for the forward primer and 279 µL for the reverse primer. They were stored at -20°C. For the PCR reactions, these stock solutions were diluted 1:10, and 1.5 µL of the dilution were used. The other components required for the PCR were 12.5 µL of Q5 High Fidelity 2X Master Mix from New England Biolabs (Ipswich, United Kingdom), 1 µL of the isolated plasmid, and 10 µL of autoclaved water. The PCR was performed under the following conditions: First, denaturation was carried out at 98°C for 3 min. This was followed by 30 cycles of denaturation at 98°C for 30 sec, primer annealing at 57°C for 45 sec, and elongation at 72°C for 1 min. The amplified DNA was separated in 0.8% agarose in TBE buffer. It was stained with 10 µL of GelRed® and visualized using the ChemDoc MP imaging system (Bio-Rad, USA).

4.8. Infection experiments with scalpel

Infection experiments through wounding of leaves were performed with *Gentiana cruciata* plantlets. The concentration of *R. rhizogenes* is usually adjusted to an OD_{600} between 0.6 and 1.0 (Zhu et al., 2024). One colony of *R. rhizogenes* strain A4 was picked and incubated overnight at 28°C and 200 rpm in 2 ml of YEB medium. The next day, 800 µL of this culture was diluted in 20 mL YEB medium. After 4 hours at 28°C and 200 rpm, the suspension had an $OD_{600\ nm}$ of approximately 0.6 and was ready for the infection experiments. Subsequently, 19 µL of a 100 mM acetosyringone stock solution were added to 19 mL of bacterial suspension so that a concentration of 100 µM was obtained. The plantlet was removed from the Hipp® jar and placed on sterile filter paper. The infection was carried out by dipping a scalpel into the bacterial suspension and then cutting three times into the surface of the leaf without cutting through it, as illustrated in figure 9 (page 19) (Santos et al., 2005). Afterwards, it was inoculated on semi-solid hormone-free 1/2MS medium and cultivated at 25°C under a photoperiod of 16 hours.

Figure 9: Infection of *G. cruciata* leaves using a scalpel dipped in bacterial suspension. The red arrows indicate the cuts.

4.9. Infection experiments through co-cultivation

Another method to induce hairy root formation is co-cultivation (Georgiev et al., 2011). 6 to 8 weeks old plantlets of *G. cruciata*, *G. lutea* and *G. asclepiadea* were used for these experiments. Hypocotyl, cotyledons, and leaves were detached from the plantlets and were temporarily stored in liquid 1/2 MS medium to prevent them from drying out. A culture of *R. rhizogenes* strain A4 adjusted to an OD_{600} of 0.6 as described previously was centrifuged at 5000 rpm for 10 min. The YEB medium was then discarded and replaced with an aliquot amount of liquid 1/2 MS. After mixing, an appropriate amount of acetosyringone was added to achieve a concentration of 100 µM. Afterwards, cotyledons, hypocotyls, and leaves were transferred to the suspension and incubated for 30 minutes with repeated shaking. Another method was sonication-assisted *Agrobacterium*-mediated transformation (SAAT) (Trick & Finer, 1997). In this method, the explants were treated with ultrasound (Elma Transsonic 460, Elma Schmidbauer GmbH, Germany) at a frequency of 35 kHz for 5, 10, or 15 seconds. Following the bacterial treatment the explants were blotted dry on sterile cellulose sheets and transferred to 60 mm Petri dishes with semi-solid 1/2 MS medium supplemented with 100 µM acetosyringone. The explants were then kept in the dark at 25°C for 2 days. Subsequently, the explants were washed in liquid 1/2 MS medium containing 300 mg L^{-1} cefotaxime sodium, blotted dry on

sterile cellulose sheets, and transferred to semi-solid 1/2 MS medium supplemented with 300 mg L^{-1} cefotaxime sodium. The explants of *G. lutea* and *G. asclepiadea* were then kept at 18°C under a photoperiod of 16 h (figure 10, page 20), the explants of *G. cruciata* were kept at 25°C under the same light regime.

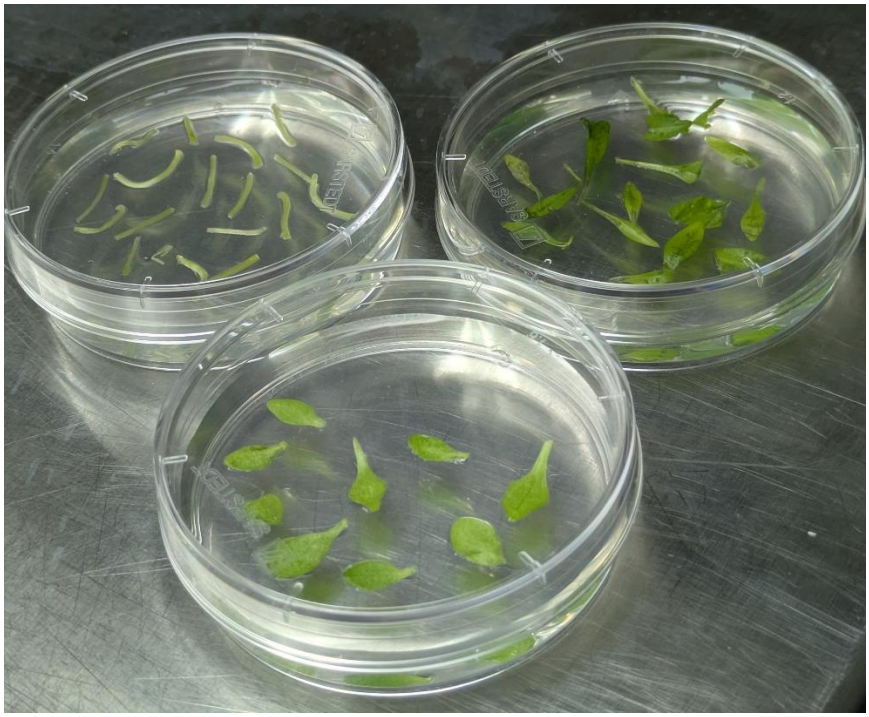


Figure 10: Co-cultivation of *G. lutea.*

4.9. Adventitious root culture

To compare the content of gentiopicroside, swertiamarin, and amarogentin in hairy roots, adventitious root cultures were established. To this purpose, roots of approx. 6-8 weeks old plantlets were transferred to 20 ml of liquid 1/2MS medium supplemented with 0.5 µM 6-benzylaminopurine (BAP) (Sigma Aldrich) and 5.0 µM 1-naphthaleneacetic acid (NAA) (Sigma Aldrich) (Momcilovic et al., 1997a). The roots were cultivated on a rotary shaker at 90 rpm in the dark for 3 weeks and were subsequently transferred to 250 ml Erlenmeyer flasks containing 50 ml of 1/2 MS medium without growth regulators, under the same conditions.

4.10. HPLC analysis

For HPLC analysis, freeze-dried roots from the adventitious root culture of *G. lutea* and *G. cruciata were* used, as well as *Gentianae radix* (KOTTAS Pharma GmbH, n.d.) powder. These were tested for the presence of the compounds gentiopicroside, swertiamarin, and amarogentin.

4.10.1. Sample preparation

The extraction of the root samples for HPLC analysis was performed according to Falcone et al. (2025). The roots were removed from the culture medium and blotted dry on cellulose sheets. The fresh weight was determined, and the roots were then transferred to paper bags, frozen, and lyophilized (Zirbus Technology GmbH, Bad Grund, Deutschland) for 48 hours. The freeze-dried roots were weighed and pulverized in a mortar and pestle. 100.0 mg of root powder of *G. cruciata*, *G. lutea* and commercially available *Gentianae radix* were extracted with 3 ml of HPLC-grade methanol for 20 min in an ultrasonic bath at room temperature. The samples were then centrifuged for 10 minutes at 5000 rpm and the supernatant was collected in a 10 ml measuring flask. This process was repeated twice. The collected solvents were diluted to 10.0 ml with MeOH. The extracts of *G. lutea* and *G. cruciata* were then concentrated. To do this, 5 ml were evaporated and stored at -20°C. Right before analysis the samples were then redissolved in 1.00 ml of methanol and centrifuged at 15000 rpm for 10 min.

4.10.2. HPLC parameters

Quantification of gentiopicroside, swertiamarin and amarogentin was carried out using an HPLC system from Shimadzu Corporation (Kyoto, Japan). The HPLC setup consisted of a DGU-20A5R degassing unit, an LC-20AT pump, a SIL-20AT HT autosampler, a CTO-20AC column oven, an SPD-M20A diode array detector, and a CBM-20A communication module. Separation was performed on a Luna® C18(2) column (250 × 4.6 mm, particle size 5 µm) supplied by Phenomenex Ltd.

(Aschaffenburg, Germany). The mobile phase A consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). Table 1, page 22 shows the HPLC gradient parameters. The flow rate was set to 1.0 ml min^{-1}, the column temperature to 30°C, and the separation was monitored at 232 nm and 272nm.

Table 1: HPLC gradient (after Falcone et al. 2025).

Time (min)	A (vol%)	B (vol%)
0.00	95	5
5.00	95	5
30.00	70	30
35.00	20	80
37.00	20	80
39.00	0	100
45.00	0	100

4.10.3 Calibration curves of standard compounds

A calibration curve had to be created based on external standards for the quantification and identification of gentiopicroside, swertiamarin, and amarogentin. Stock solutions were prepared by dissolving 3.00 mg of each of the standard compounds in 3.00 ml of methanol. Subsequently, a dilution series was prepared to obtain a total of 10 different concentrations. The concentrations were 1000 µg mL^{-1}, 500 µg mL^{-1}, 100 µg mL^{-1}, 50 µg mL^{-1}, 25 µg mL^{-1}, 10 µg mL^{-1}, 5 µg mL^{-1}, 2.5 µg mL^{-1}, 1 µg mL^{-1} and 0.5 µg mL^{-1}. The samples were measured three times each with an injection volume of 10 µL.

5. Results

5.1. Germination of *Gentiana* species

In standard conditions (without GA_3 and at a temperature of 25°C), no satisfactory germination occurred in any species during the first weeks of the study. A series of experiments was initiated using the parameters described in chapters 4.3. and 4.4.. The germination of *G. purpurea* was not successful under any of the conditions tested. This series of experiments was conducted using a total of 400 *G. purpurea* seeds.

To determine the optimal conditions for *G. cruciata*, GA_3 pretreatment and incubation at 4°C were applied at the intervals described. The 24-hour GA_3 pretreatment at 500 mg L^{-1} resulted in germination after approximately 11 days. The seeds that were kept at a cold temperature for 2 to 6 weeks also germinated after being transferred back to the culture room at 25°C. However, the germination rate for these was lower and inconsistent. In contrast, the GA_3 treatment showed a germination rate of nearly 100%. The following conditions were established: 24-hour GA_3 pretreatment at 500 mg L^{-1}, surface sterilization with 10% NaClO for 10 minutes, and germination on 1/2 MS medium at 25°C. Under these conditions, seedlings emerged after 3 weeks (figure 11, page 24) and plantlets with leaves were available after 4 to 6 weeks (figure 12, page 24). Approximately 75% of these were suitable for further studies in terms of growth and morphology.

Figure 11: 20 days old *G. cruciata* seedlings.

Figure 12: 6 weeks old *G. cruciata* plantlets.

Since the seeds from *G. asclepiadea* were special GOLD NUGGET SEED®, they should not have required treatment with GA_3 or cold stratification. At 25°C and without GA_3, the seeds germinated only sporadically. When they did germinate, they did not grow properly, lost their colour, and died. The die-off began at the root, which first turned black. Therefore, a series of experiments was started using GA_3 and cultivating at 25°C and without GA_3 at 18°C. Despite the GA_3 treatment at 25°C, this did not change the germination behaviour. The seeds kept at 18°C germinated after 11 days and were large enough for further experiments after 6 weeks. The percentage of seedlings that were usable was significantly lower than that of *G. cruciata*. As shown in figure 13 (page 25), many seedlings eventually bleached out and died off. The seedlings that were not usable were either underdeveloped, or the seeds failed to germinate at all. Figure 14 (page 25) shows healthy seedlings of *G. asclepiadea.* The following conditions were used for the subsequent experiments: No GA_3 treatment, surface sterilization with 10% NaClO for 10 minutes, and germination on 1/2 MS medium at 18°C.

Figure 13: *G. asclepiadea,* loss of colour after 10 weeks.

Figure 14: 8 weeks old *G. asclepiadea* plantlets.

Initially, germination of *G. lutea* seeds was unsuccessful. In the first experiments the seeds were kept at 25°C, and surface sterilization with various concentrations of NaClO (10–25%) as well as exposure times of 5–15 minutes were tested. Subsequently, an experiment with GA_3 was also conducted. None of these altered parameters resulted in successful germination. Those that did germinate as shown in figure 15 (page 26) died shortly afterwards or lost their colour and were unusable for further investigations. Another series of experiments was initiated as described in chapter 4.3. The aim was to determine whether temperature or the nutrient medium had an influence on germination. The seeds germinated after 11 days. Seedlings were ready after 3–4 weeks (figure 16, page 26), and plantlets with their first leaf were available after approximately 6 weeks. Out of 70-80 seeds, approximately 40% had grown into usable seedlings. The results clearly showed, as seen in figure 17 (page 26), that temperature has a significant influence on the germination. The following conditions were used for further experiments: No GA_3 treatment, surface sterilization with 10% NaClO for 10 minutes, and germination on 1/2 MS medium at 18°C.

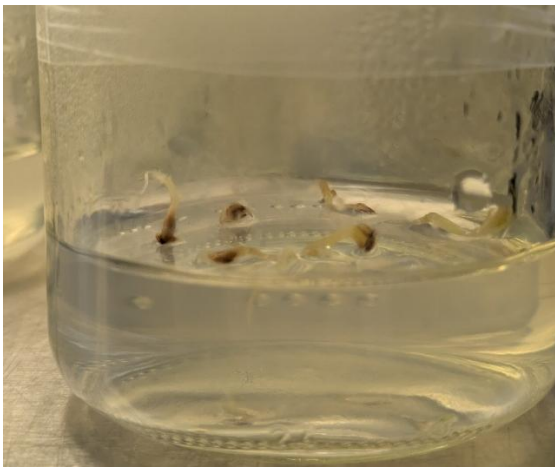


Figure 15: 4 weeks old *G. lutea*, dieback shortly after germination.

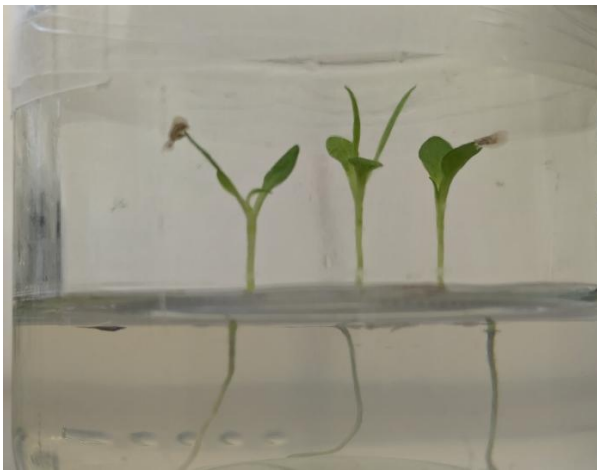


Figure 16: 4 weeks old healthy *G. lutea* seedlings.

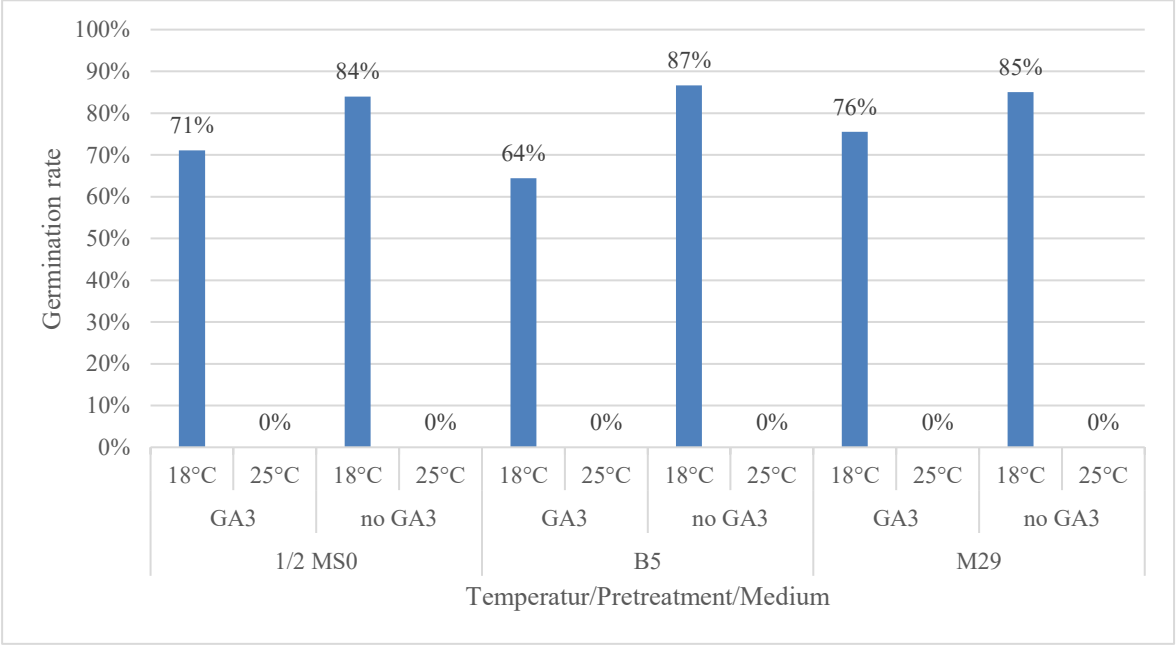


Figure 17: Influence of temperature, GA_3 treatment, and nutrient medium on the germination rate of *G. lutea.*

5.2. Growth of *R. rhizogenes* strain A4

As shown in figure 18 (page 27), a growth curve based on optical density (OD_{600}) measurements was recorded to determine the optimal incubation time. Bacteria were prepared as described in chapter 4.5. The bacterial cultures were monitored at various time points (90, 150, 180, 240, 270, and 300 minutes) to analyse their progression throughout the growth phases. The recorded OD_{600} values rose steadily from 0.24 at 90 minutes to 0.91 at 300 minutes, indicating continuous cell proliferation.

The culture reached an OD_{600} value of approximately 0.6–0.7 between 240 and 270 minutes, which corresponds to the mid-log phase, typically considered optimal for further experiments. Based on these results, the optimal incubation time was set at approximately 4 to 4.5 hours.

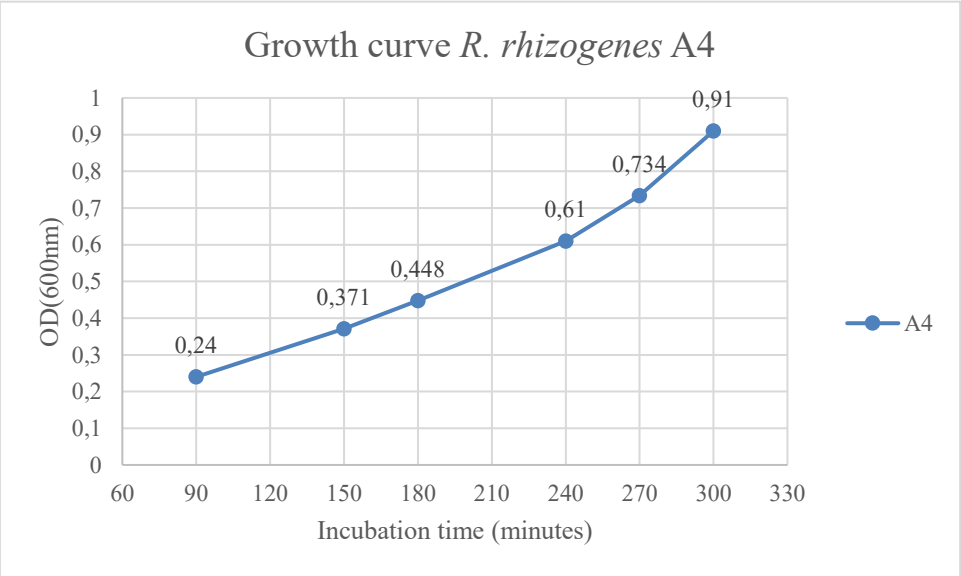


Figure 18: Growth curve of *R. rhizogenes* A4.

5.3. Detection of *rolB* gene

The presence of the *rolB* gene, and thus the Ri plasmid, was verified using PCR amplification. Without this plasmid, the bacterium would not be able to induce hairy root growth (Liu et al., 2025). As described in Chapter 4.6, the bacterial strains A4 and ATCC15834 were tested. Amplification of the target gene was successfully performed for both strains. The PCR product was visualized by gel electrophoresis. As can be seen in figure 19 (page 28), a 1-kb DNA ladder is present in Lane 1. Lane 2 shows the *R. rhizogenes* A4 *rolB*, and Lane 3 shows the *R. rhizogenes* ATCC15834 *rolB*. Accordingly, successful induction of hairy root growth should be possible with these bacterial strains.

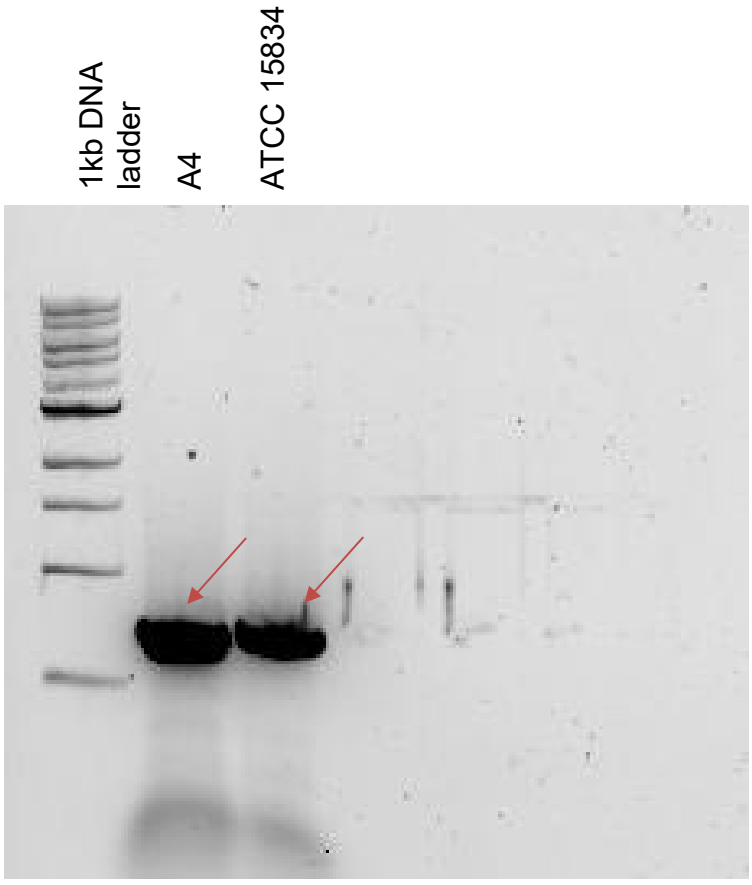


Figure 19: Detection of *rolB* gene of A4 and ATCC 15834.

5.4. Establishment of hairy root cultures

One aim of this study was to infect the seedlings with *R. rhizogenes* and induce the formation of hairy roots. To do this, the leaves were lightly cut with a scalpel, which had previously been dipped in a bacterial suspension, without cutting all the way through. Since a suitable method for obtaining seedlings had already been established for *G. cruciata*, the experiments were started with this species. The cuts were made in the first leaf after the seedling had reached an age of approximately 6 weeks (figure 20, page 29). A total of 50 seedlings was infected with the scalpel. According to Hayta et al. (2011), hairy roots should appear 16–25 days after direct infection. Upon close observation, however, no hairy root growth was detected even after 4 weeks in this series of experiments. Therefore, another series of experiments was initiated to identify a more efficient method of infection.

Figure 20: 6 weeks old *G. cruciata* seedlings after infection with scalpel.

Once the optimal germination conditions (18°C) for the other two species, *G. lutea* and *G. asclepiadea*, had been identified, infection experiments were conducted through co-cultivation. Co-cultivation was a slightly more complex method, as the bacterial culture was centrifuged to replace the YEB medium with 1/2 MS medium. The OD_{600} was not always 0.6 after 4 hours but fluctuated between 0.55 and 0.61. Since it was not possible to wait longer than 4 hours and take another measurement

due to technical constraints, the medium volume for the re-suspension was adjusted accordingly to achieve an OD_{600} of 0.6.

The seedlings were dissected into cotyledons, hypocotyls, and leaves, as shown in figure 21 (page 30). *G. cruciata* grew most uniformly over the 6 weeks and was the easiest to dissect. The cotyledons were large enough to be cut cleanly. In contrast, the cotyledons of *G. asclepiadea* were very small and difficult to handle. Furthermore, *G. asclepiadea* exhibited irregular growth, resulting in seedlings of varying sizes. Like *G. cruciata*, *G. lutea* showed more consistent growth and was easier to handle. The explants were inoculated in the bacterial suspension, treated with ultrasound for varying durations (0, 5, 10, and 15 seconds) and subsequently cultivated for 48 hours. Ultrasound treatment causes minor damage to the explants, thereby increasing the efficiency of the infection.

Figure 21: 8 weeks old seedlings of *G. lutea* dissected into cotyledons, hypocotyls and leaves.

One week after co-cultivation, the cotyledons of all species started to turn brown (figure 22, page 31). The hypocotyls of *G. lutea* and *G. asclepiadea* also turned brown. In contrast, the hypocotyls of *G. cruciata* remained viable, exhibited adventitious shoot formation, and resumed growth (figure 23, page 31). The co-cultivation involved a total of 449 seedlings. Of these seedlings, 217 were *G. cruciata*, 106 were *G. asclepiadea* and 126 were *G. lutea*. There was no hairy roots growth in any of these tests.

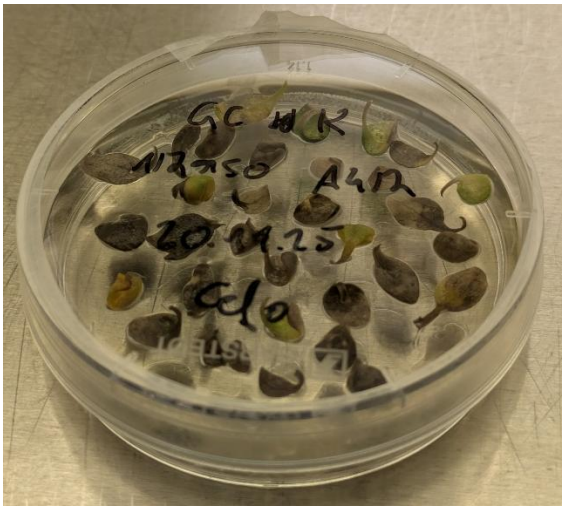


Figure 22: *G. cruciata* cotyledons, no hairy roots growth after 6 weeks.

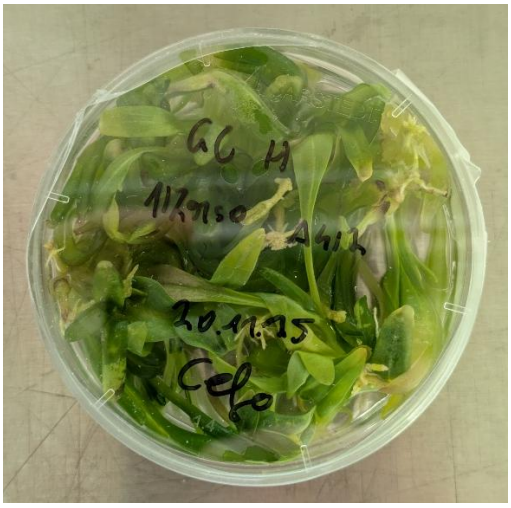


Figure 23: *G. cruciata* hypocotyls, no hairy roots growth after 6 weeks.

5.5. Establishment of adventitious root cultures

Adventitious root cultures were established for further studies in order to compare the levels of gentiopicroside, swertiamarine, and amarogentin with those in hairy root cultures. As described in chapter 4.9 on page 20, the roots of the 6–8-week-old seedlings were cut off and transferred to liquid medium. Subsequently the roots were subcultured every 3 weeks.

The adventitious root cultures of *G. asclepiadea* did not grow sufficiently during the observation period despite the addition of NAA and BAP and could not be used further. Five adventitious root culture lines were established for *G. cruciata* and four for *G. lutea*. After two months of cultivation, the roots were freeze-dried, pulverized and weighed.

The dry weight and fresh weight for *G. lutea* are shown in figure 24 (page 32), and those for *G. cruciata* in figure 25 (page 32). What is apparent is the highly irregular growth and, consequently, the high standard deviation. Despite the same time period and identical treatment, there were significant differences in mass even within the same species. Figure 26 (page 33) shows the adventitious root culture of *G. cruciata*, while figure 27 (page 33) displays that of *G. lutea*. The visible differences in colour and thickness of the roots are also noticeable.

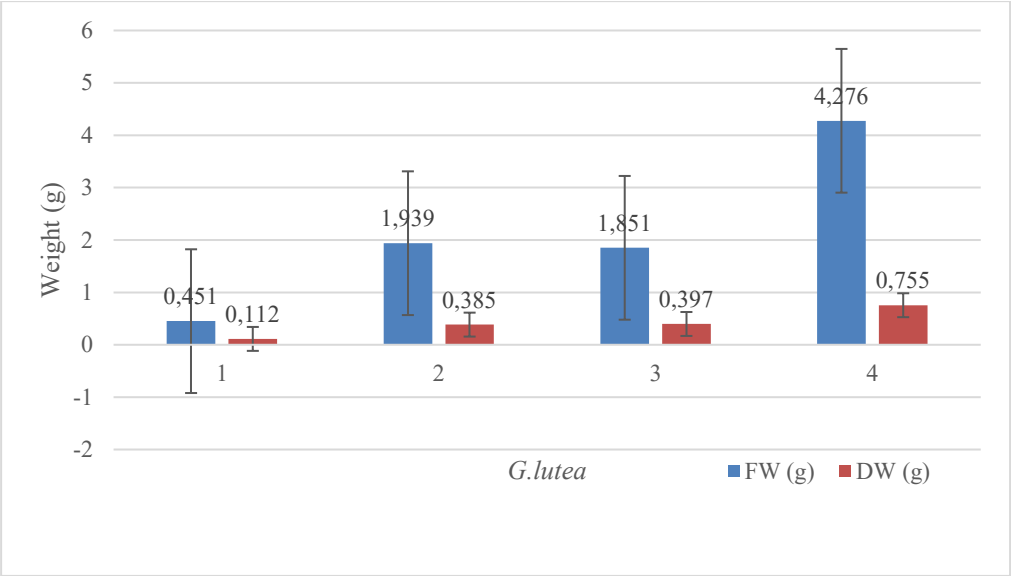


Figure 24: Adventitious root cultures of *G. lutea*, fresh weight (FW) and dry weight (DW) (n=4)

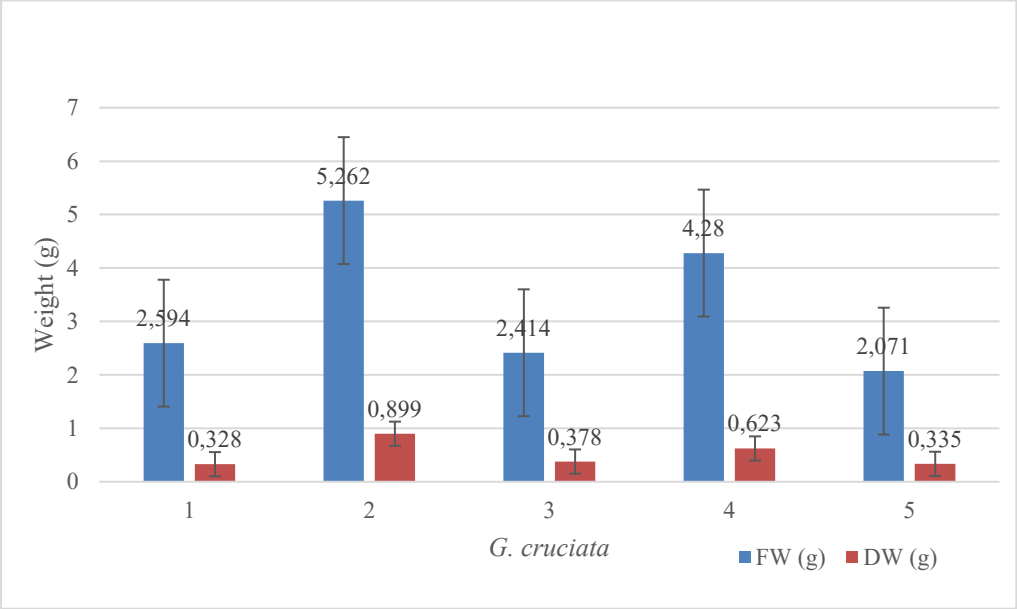


Figure 25: Adventitious root cultures of *G. cruciata*, fresh weight (FW) and dry weight (DW) (n=5)

Figure 26: Adventitious root culture of *G. cruciata.*

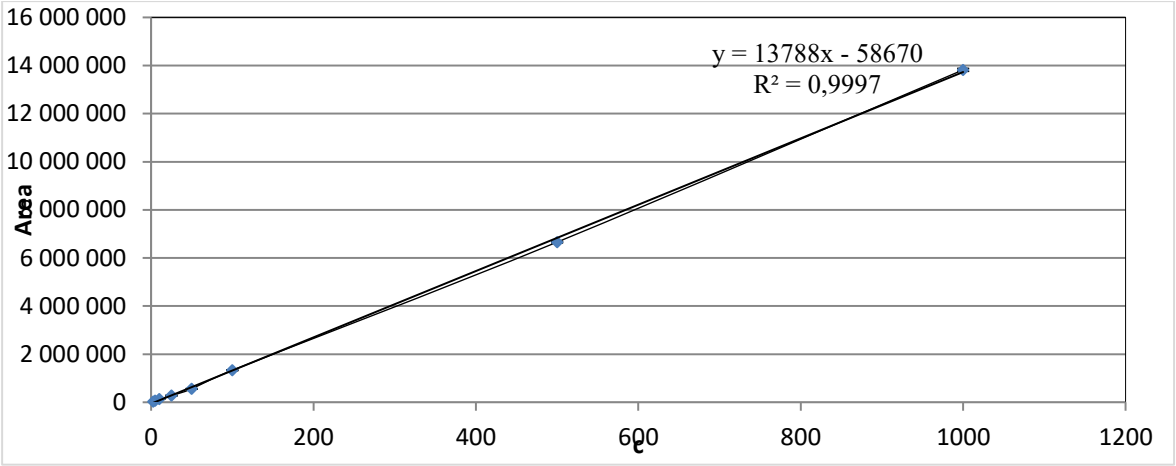
Figure 27: Adventitious root culture of *G. lutea.*

5.6. HPLC analysis of adventitious root cultures

To validate the HPLC method for the analysis of *Gentiana* samples, external standards of gentiopicroside, swertiamarin, and amarogentin were used for identification and quantification. Calibration curves were generated for each compound to ensure the suitability of the analytical method (figures 28 and 29, page 34, and figure 30, page 35). The calibration curve was prepared as described in chapter 4.10.3.. According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (2023), the Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined based on the standard deviation of the response (σ) at low concentrations and the slope (S) of the corresponding calibration curve. LOD and LOQ were calculated using the following equations:

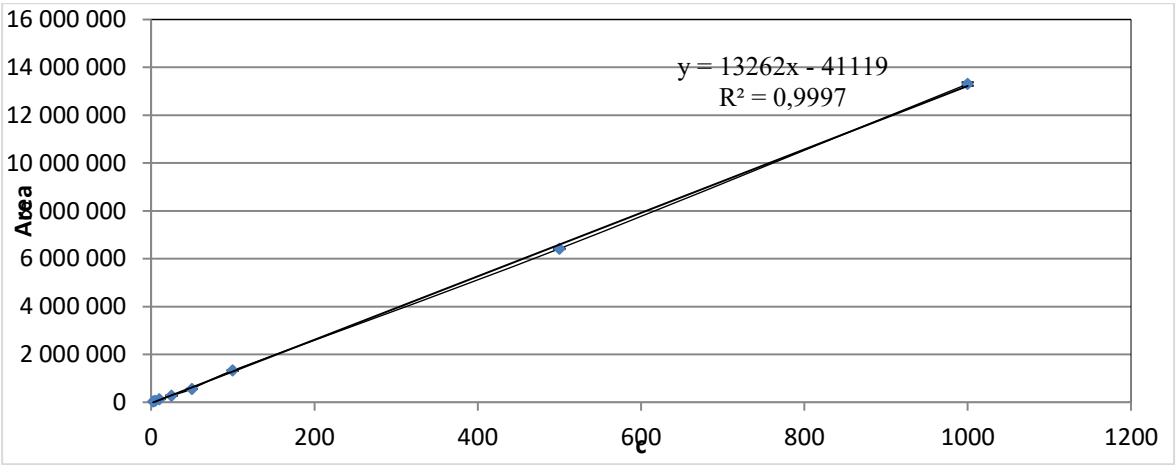
$$LOD = \frac{3.3 * \sigma}{S}$$

$$LOQ = \frac{10 * \sigma}{S}$$



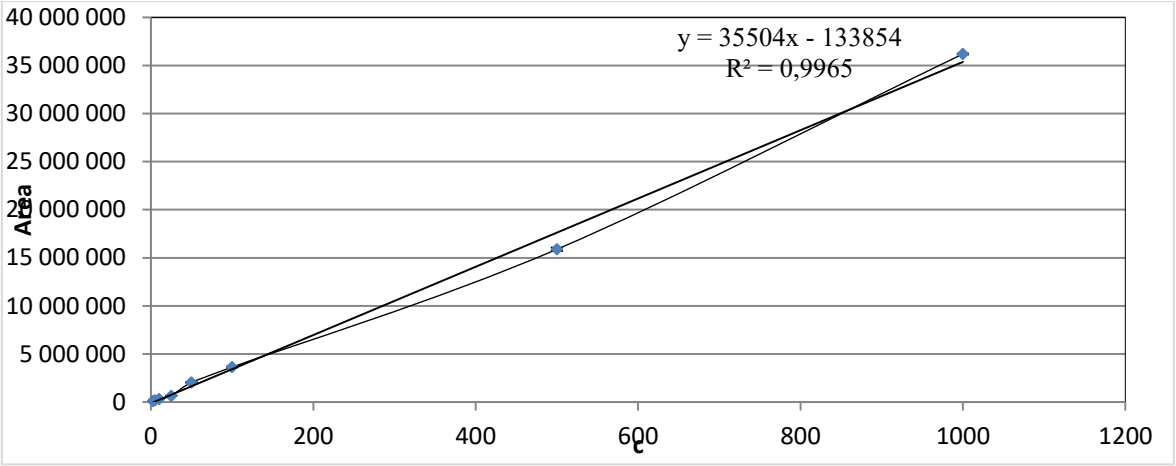
LOD =	2,51	µg/ml
LOQ =	7,61	µg/ml

Figure 28: Calibration curve of gentiopicroside.



LOD =	2,31	µg/ml
LOQ =	6,99	µg/ml

Figure 29: Calibration curve of swertiamarin.



LOD =	1,94	µg/ml
LOQ =	5,89	µg/ml

Figure 30: Calibration curve of amarogentin.

The HPLC method was used to quantify the content of gentopicroside, swertiamarin, and amaraogentin in the samples. The samples were prepared as described in chapter 4.10.1. (page 21). The substances were detected at two wavelengths: 232 nm was ideal for detecting swertiamarin and amarogentin (Mustafa et al., 2015), and 272 nm (Sobot et al., 2020) caused less interference to the quantification of gentiopicroside. The presence of the target compounds was confirmed based on their characteristic retention times. Swertiamarin was detected at approximately 20.7 min (232 nm), while gentiopicroside eluted at approximately 22.8 min (272 nm) and amarogentin was detected at 35.5 min (232 nm).

As can be seen in figure 31 (page 36) and figure 32 (page 36), no distinct peak was observed at the retention time of gentiopicroside in samples GL-R1 and GL-R2 (*G. lutea*).

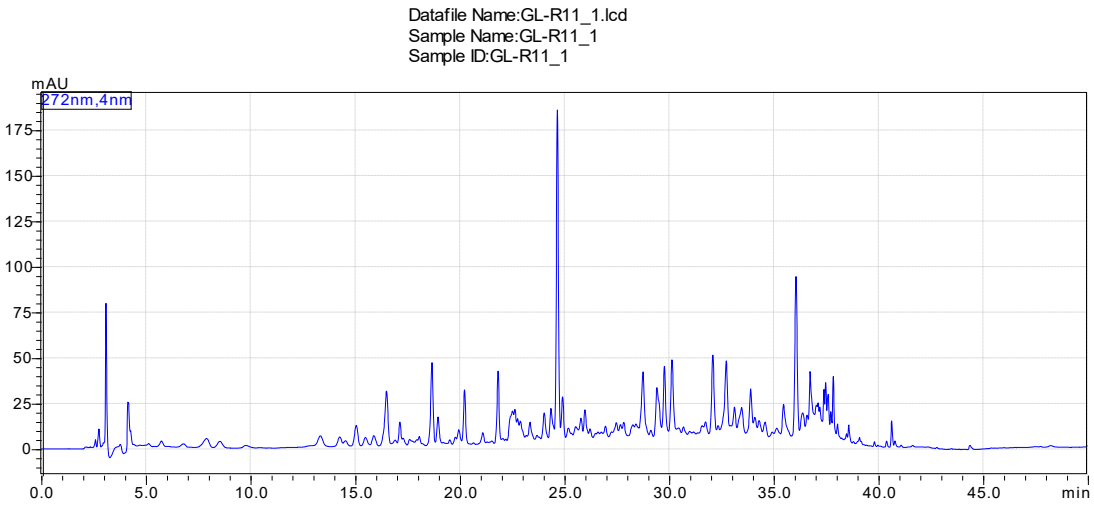


Figure 31: HPLC chromatogram of sample GL-R1 (*G. lutea*).

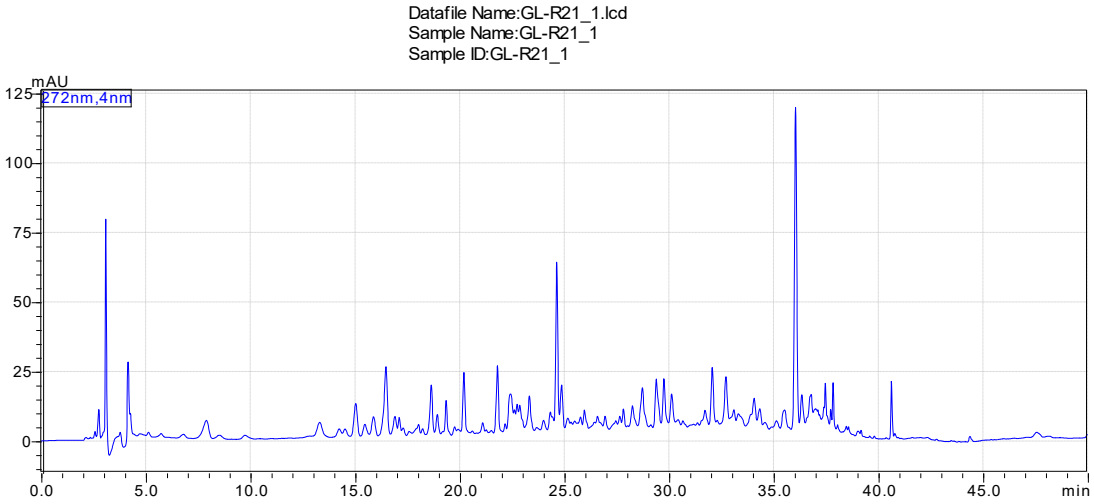
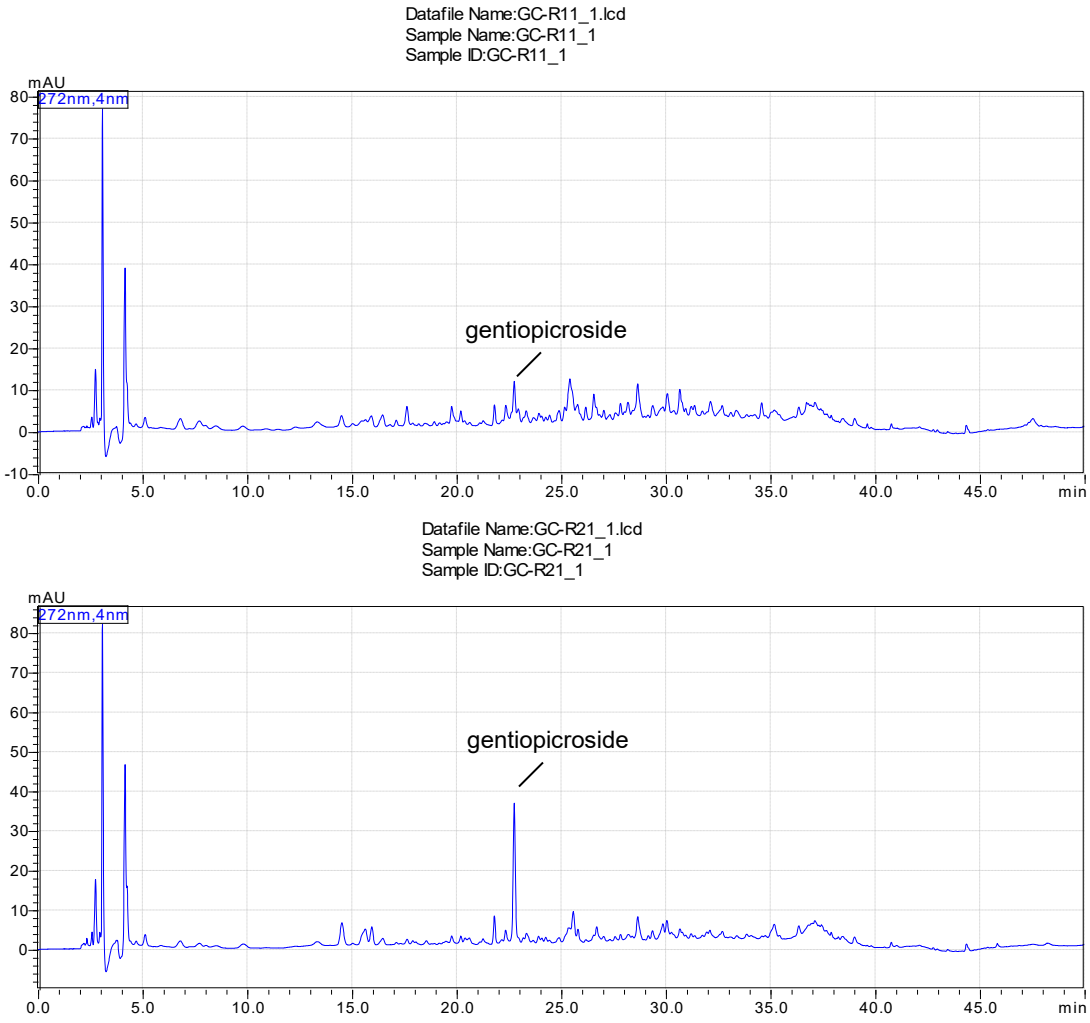


Figure 32: HPLC chromatogram of sample GL-R2 (*G. lutea*).

Table 2 (page 37) summarizes the quantified target compounds across the analysed samples. Gentiopicroside was quantifiable in every *G.cruciata* sample (GC-R1, GC-R2 and GC-R3) (figure 33, page 37 and 38), in *Gentianae radix* (G-R) (figure 34, page 38), and in one *G. lutea* (GL-R3) (figure 35, page 39) sample.

Table 2: Content and standard deviation of gentiopicroside, swertiamarin and amarogentin in analysed samples.

Sample	Gentiopicroside (%)	Swertiamarin (%)	Amarogentin (%)
GL-R3	0.1136 ± 0.003	-	-
GC-R1	0.0195 ± 0.0004	-	-
GC-R2	0.0556 ± 0.008	-	-
GC-R3	0.0334 ± 0.019	-	-
G-R	2.6506 ± 0.03	0.0708 ± 0.0003	-



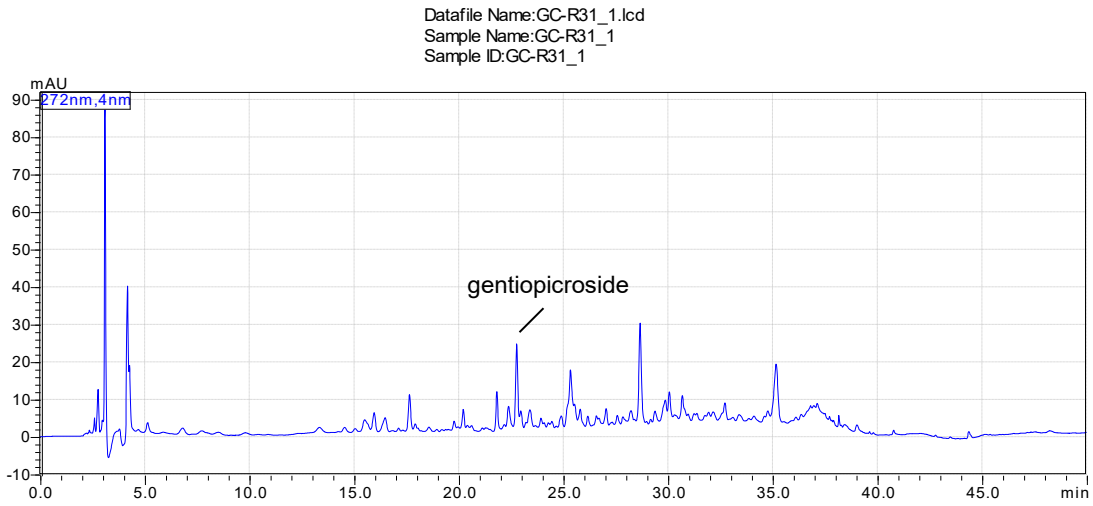


Figure 33: HPLC - UV chromatogram of *G. cruciata* samples GC-R1, GC-R2 and GC-R3 recorded at 272 nm. The peak representing gentiopicroside is detected at a R_t of approximately 22.8 minutes.

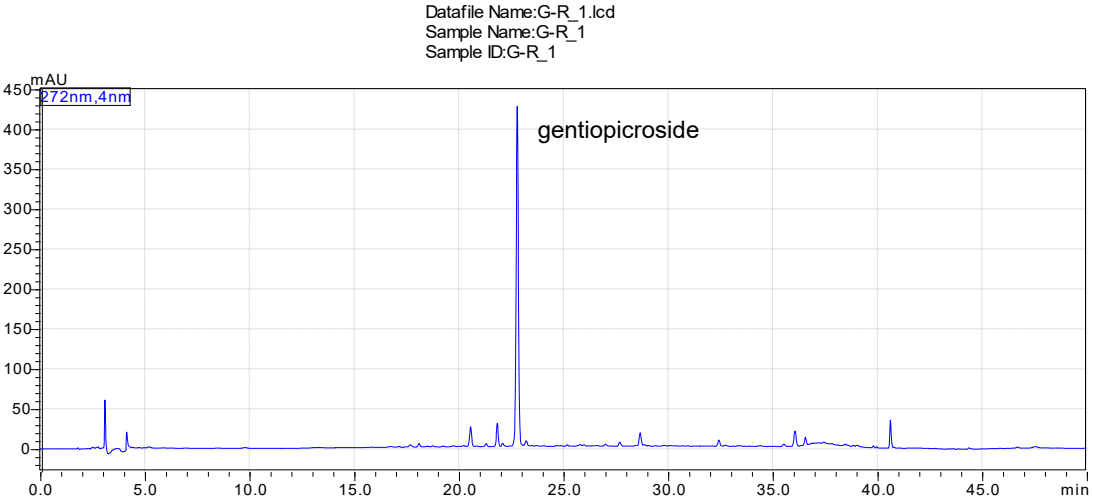


Figure 34: HPLC - UV chromatogram of *Gentianae radix* recorded at 272 nm. The peak representing gentiopicroside is detected at a R_t of approximately 22.8 minutes.

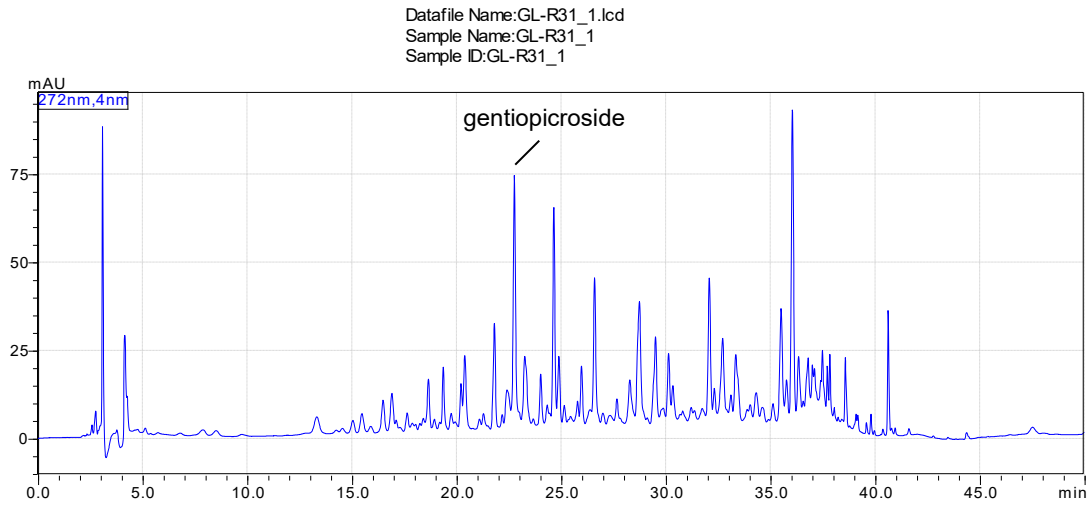


Figure 35: HPLC - UV chromatogram of *G. lutea* sample GL-R3 recorded at 272 nm. The peak representing gentiopicroside is detected at a R_t of approximately 22.8 minutes.

Amarogentin was not quantifiable in any of the samples, and swertiamarin was not quantifiable in any of the adventitious root culture samples. The content of gentiopicroside was 23-fold higher in *Gentianae radix* (2.65%) compared to the highest gentiopicroside content in the adventitious root culture (GL-R3 0.11%). Swertiamarin was also quantifiable only in *Gentianae radix* (0.07%) (figure 36, page 40). While the first graph (figure 37, page 40) shows the gentiopicroside and swertiamarin (SW) content of adventitious root cultures in direct comparison to *Gentianae radix*, the second graph (figure 38, page 41) uses a rescaled y-axis to make the significant differences between samples with lower concentrations more clearly visible. Note that G-R and G-R (SW) represent the gentiopicroside and swertiamarin content of a single *Gentianae radix* sample.

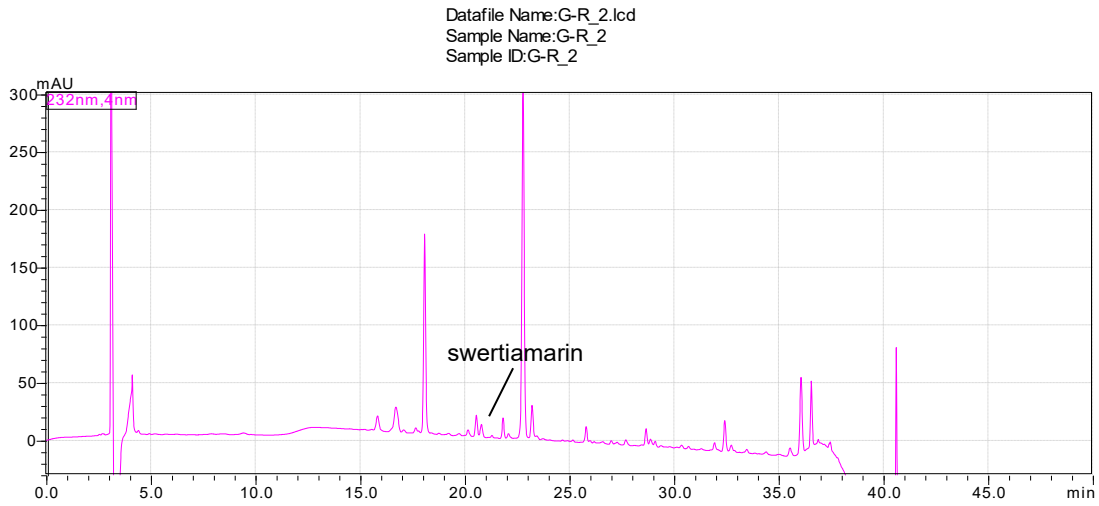


Figure 36: HPLC - UV chromatogram of *Gentianae radix* recorded at 232 nm. The peak representing swertiamarin is detected at a R_t of approximately 20.7 minutes.

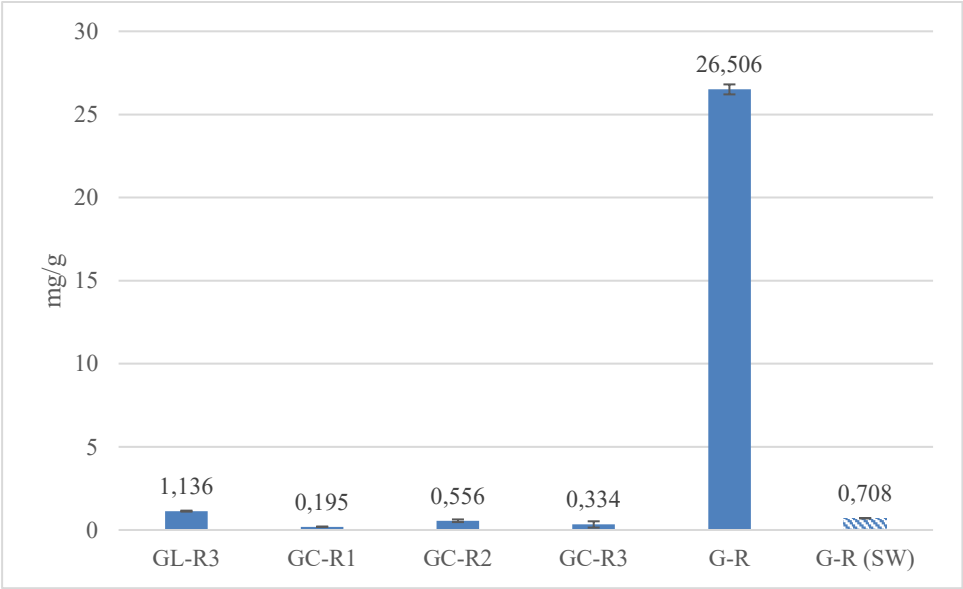


Figure 37: Quantitative distribution of gentiopicroside and swertiamarin (mg/g) across all analysed samples. The error bars indicate the standard deviation (n = 3).

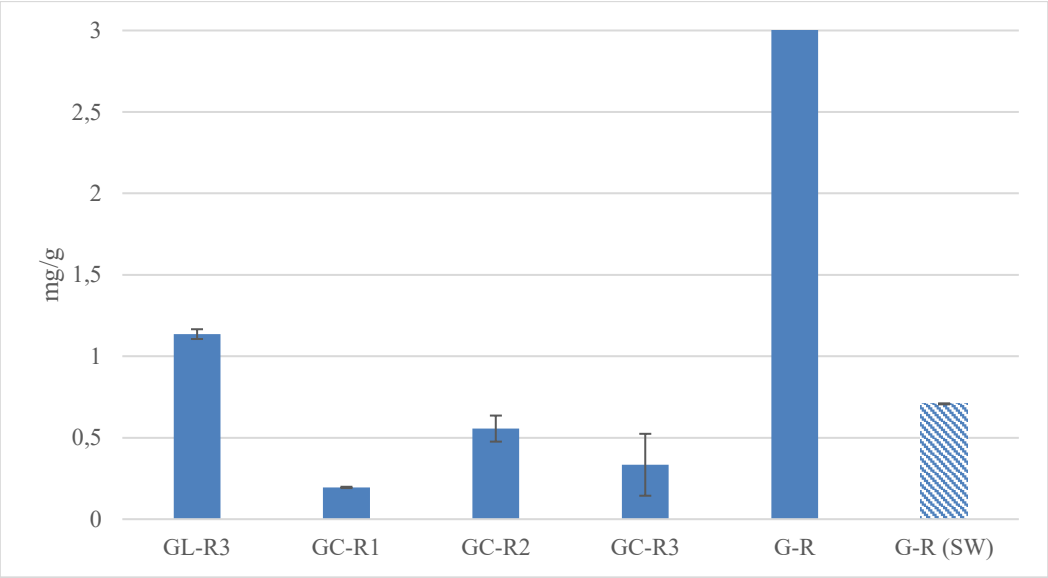


Figure 38: Rescaled quantitative distribution of gentiopicroside and swertiamarin (mg/g) to highlight differences among the lower-concentration samples. Error bars indicate the standard deviation (n = 3).

6. Discussion

The aim of this study was to establish hairy root cultures of four species of the genus *Gentiana*. There are only a few reports in the literature regarding the successful induction of hairy root cultures in the species *G. lutea*, *G. cruciata*, *G. purpurea*, and *G. asclepiadea* (Hayta et al., 2011; Momčilović et al., 1997b). Driven by the substantial global market demand for roots of *Gentiana* species, establishing sustainable production methods continues to be a key priority in contemporary research (Falcone et al., 2025; González-López & Casquero, 2014; Xu et al., 2017). In the first part of the project, it was important to get the seeds to germinate. The availability of healthy seedlings was a major problem at the start of the study.

There is little literature on the *in vitro* germination of *G. purpurea*. Momčilović et al. (1997a) describe successful germination after a GA_3 treatment. The company Jelitto®, from which our seeds were purchased, indicates a germination temperature of +18 to +22°C and well-moistened conditions during the first 2–4 weeks. Afterwards, the seeds should be exposed to a cold temperature between –4 and +4°C. After this cooling period, the best temperature range is then between +5 and +12°C (Jelitto Staudensamen GmbH, n.d.).
Since it was technically not feasible to maintain a growth room at a temperature between 5°C to 12°C, the seeds had to be kept at 18°C. The elevated temperature may be one reason why *G. purpurea* did not germinate successfully. Germination of the other species was also a problem, which caused a substantial time investment. Because the pharmaceutical drug *Gentianae radix* consists of the dried underground parts of *Gentiana lutea* (*Europäisches Arzneibuch*, 2023), this species was of great importance for this study. For this reason, a separate series of experiments was conducted. The change in temperature from 25°C to 18°C was critical for the germination success of the species *G. lutea* and *G. asclepiadea*. The germination at lower temperatures, as described in chapter 5.1., illustrates the sensitivity of these species. It took considerable time for the seedlings to be ready for the inoculation experiments (4–6 weeks for *G. cruciata* and 6–8 weeks for *G. lutea* and *G. asclepiadea*). One way to accelerate growth prior to inoculation with the bacterium would be to use a medium containing BAP and NAA (Demirci et al.,

2024). *G. cruciata* showed the highest germination rate (100%) upon treatment with GA_3 and produced the highest proportion of viable seedlings (75%), making this species a candidate as the most practical option for further studies.

In total, 499 seedlings were infected with *Rhizobium rhizogenes* strain A4 in this study. Other studies have reported the formation of hairy roots after 4 weeks (Hayta et al., 2011; Momčilović et al., 1997b; Zhang et al., 2010). Nevertheless, in our experiments none of the seedlings showed any hairy root growth after this period. The explants were also kept under the same conditions for an additional 8 weeks to check for any delayed growth, but no hairy root formation was observed during that time either. The scalpel-based infection method turned out to be particularly difficult for *G. cruciata*, as the leaves of the plantlets were very thin. Hayta et al. (2011) reported a higher success rate with the stem than with the leaves: In this study, the plants were inoculated with *R. rhizogenes* by puncturing the internodes. The explants were then placed in a bacterial suspension with an OD_{600} of 1.0 for 30 minutes. This study successfully combined the scalpel method with co-cultivation. Due to the lack of results and the difficulty of using a scalpel on the thin leaves, the method used in our study was changed to co-cultivation. Since the procedure was easier to handle and thus allowed for more consistent results, this method appeared more promising. Georgiev et al. (2011) demonstrated increased efficiency in co-cultivation of *Verbascum xanthophoeniceum* Griseb. through sonication-assisted *Rhizobium rhizogenes*-mediated transformation. Since the ultrasound allowed the explants to be wounded in a controlled manner, reproducibility was also improved. *Verbascum* leaves are thicker than those of *Gentiana* species. Therefore, we adjusted the exposure time to ultrasound to 5, 10, and 15 seconds. However, as described in chapter 5.4. co-cultivation was also unsuccessful. This could be due to the following reasons:

The type of explant and the age of the seedlings can influence the transformation efficiency of *R. rhizogenes* (Hayta et al., 2011; Tiwari et al., 2007). Seedlings that are too old can exhibit reduced cell division rate. On the other hand, Momčilović et al. (1997a) described that shoots only a few centimetres long responded by producing hairy roots, while shorter ones failed to respond. The duration of immersion of the explants in the *R. rhizogenes* suspension also has an influence.

Zhang et al. (2010) describe successful hairy root induction in *G. macrophylla* Pall after 10 min, 1 h, and 2 h of immersion in *R. rhizogenes* suspension. This study also describes more successful induction following pre-cultivation of the explants on MS medium with 1 $mg \cdot L^{-1}$ BAP. The strain of *R. rhizogenes* may also have had an additional influence. Vinterhalter et al. (2015) report higher efficiency in *G. dinarica* when using ATCC 15834 as transformation strain compared to A4. Tiwari et al. (2007) on the other hand, found no successful transformation with strain ATCC 15834 in *G. macrophylla*, but did achieve hairy root formation with strain R1000. Hayta et al. (2011) however, reported no significant differences in transformation frequency between the strains A4, ATCC 15834, R1000, and 8196 in *G. cruciata*. For further studies, it would be useful to conduct transformation experiments with strains other than A4.

We could successfully establish adventitious root cultures which were grown using the growth regulators NAA and BAP. Despite these growth-promoting substances, growth was very slow in all species. The growth of *G. asclepiadea* roots was too limited to yield sufficient root material for extraction and analysis, while enough root material of *G. lutea* and *G. cruciata* was obtained. However, the root cultures differed remarkably in appearance even within the same species, particularly in root thickness and colour. As shown in the HPLC analysis, the commercial drug *Gentianae radix* yielded the highest content of gentiopicroside (26.5 mg/g) and a quantifiable amount of swertiamarin (0.7 mg/g). In a study by Falcone et al. (2025) a gentiopicroside content of 40 mg/g and a swertiamarin content of 3 mg/g were measured. Aberham et al. (2007) reported similar results, with gentiopicroside ranging from 31–72 mg/g and swertiamarin from 3.3–6.3 mg/g. In contrast, the gentiopicroside content of the adventitious roots in our study was significantly lower (sample GL-R3: 1.14 mg/g). Swertiamarin and amarogentin were not even quantifiable. This is consistent with other studies, such as that described by Aday Kaya et al. (2025), where gentiopicroside was even below the detection limit, but swertiamarin was present at levels of 0.04–0.07 mg/g and amarogentin at concentrations of 0.4–1.0 mg/g. Interestingly, all HPLC chromatograms of the *G. lutea* adventitious root cultures (GL-R1 to GL-R3, figures 31, 32 page 36 and figure 35 page 39) displayed a prominent, yet unidentified peak at approximately 36 min (272 nm). Aberham et al. (2007) and Mustafa et al. (2015) reported that xanthones

such as gentisin, isogentisin, and their corresponding glycosides exhibit longer retention times than gentiopicroside and swertiamarin due to their lower polarity and typically elute near amarogentin. In their studies, gentiosides and xanthone aglycones were consistently prominent in *Gentiana* samples. Based on these findings, it is highly plausible that the peak observed in the present chromatograms corresponds to a xanthone characteristic of *G. lutea* root secondary metabolism. To clearly determine the identity of this compound, complementary LC–MS analysis would be required.

Unoptimized *in vitro* cultures typically exhibit significantly lower or more irregular accumulation of secondary metabolites than intact, field-grown plants, which generally maintain much higher and more stable metabolite concentrations (Murthy et al., 2014). It can be concluded that the content of gentiopicroside, swertiamarin, and amarogentin is strongly influenced by the plant's developmental stage and growth environment (Hayta et al., 2011). Another possible reason could be that some of the genes involved in the biosynthesis of gentiopicroside are more abundant in the leaves. As a result, gentiopicroside may be produced in greater quantities in the shoots and subsequently transported to the roots. In this context, Aday Kaya et al. (2025) investigated the effect of the elicitor methyl jasmonate on adventitious root cultures of *G. lutea*. It was found that this had no positive effect on the gentiopicroside content. Elicitation utilizes artificial stress factors to trigger defence mechanisms in medicinal plants, thereby upregulating the biosynthetic pathways responsible for the accumulation of secondary metabolites such as gentiopicroside (Rahmat & Kang, 2019). Other biotic and abiotic elicitors could be tested in further studies to increase the gentiopicroside and also swertiamarin and amarogentin content in adventitious roots of *G. lutea* and *G. cruciata*.

Further studies should focus on methods for successful germination of *G. purpurea* seeds. This could for example include cold stratification at 4°C, followed by incubation at temperatures between 5°C and 12°C. A temperature difference of 6–7°C (12°C to 18°C) may have been too great, as seen in the case of *G. lutea* and *G. asclepiadea*. Since the seeds of *G. lutea* and *G. asclepiadea* were specially pretreated by the company Jelitto® (GOLD NUGGET SEED®), an additional stratification was not required for these species. To ensure faster growth of the

seedlings, they could be transferred to a medium containing growth regulators after successful germination. Regarding the induction of hairy root formation, even though co-cultivation is a more standardized and comparable method than the scalpel method, working with the thin leaves and cotyledons is problematic. Multiple steps to transfer the leaves might cause excessive damage. One improvement could be to pour the bacterial suspension with the explants through a mesh. This would prevent the explants from being crushed by spoons or tweezers and treated too roughly. Another option would be to perform co-cultivation with entire shoots. This would reduce the risk of causing excessive damage to the small individual plant parts. The *R. rhizogenes* strain A4 may not be suitable for successful induction of hairy roots in the *Gentiana* species used in our studies. Experiments with other strains, such as ATCC 15834, R1000, or 8186, could be an option. In addition, the OD_{600} of 0.6 may have been too low. A series of experiments with an OD_{600} between 0.6 and 1.0 could be considered. In summary, due to the limited number of studies, a reliable method for inducing hairy roots in the species studied, namely *G. lutea*, *G. cruciata*, *G. asclepiadea*, and *G. purpurea*, is not yet available. Further research focusing on the parameters mentioned above is definitely necessary in order to achieve successful hairy root induction.

7. References

A. Salim, S., A. Abed, A., Akram, M., & Laila, U. (2021). Plant Tissue Cultural Techniques to Increasing the Production of Phytochemicals from Medicinal Plants: A Review. *Plant Arch. 21*: 1224–1229. https://doi.org/10.51470/PLANTARCHIVES.2021.v21.S1.193

Aberham, A., Schwaiger, S., Stuppner, H., & Ganzera, M. (2007). Quantitative analysis of iridoids, secoiridoids, xanthones and xanthone glycosides in *Gentiana lutea* L. roots by RP-HPLC and LC–MS. *J. Pharm. Biomed. Anal. 45*(3): 437–442. https://doi.org/10.1016/j.jpba.2007.07.001

Aday Kaya, A. G., Albayrak, İ., Demirci, T., Deveci, H., & Baydar, N. G. (2025). MeJA Changes Root Growth, Iridoid, Xanthone, and Secoiridoid Production, as well as Gene Expression Levels in Root Cultures of Endangered *Gentiana lutea* and *Gentiana boissieri*. *J. Plant Growth Regul. 44*(1): 295–315. https://doi.org/10.1007/s00344-024-11478-6

Antoniadi, L., Bartnik, M., Angelis, A., Wawruszak, A., Halabalaki, M., Kukula-Koch, W., & Skaltsounis, L. A. (2023). Gentiopicroside—An Insight into Its Pharmacological Significance and Future Perspectives. *Cells 13*(1): 70. https://doi.org/10.3390/cells13010070

Blaschek, W., Hilgenfeldt, U., Holzgrabe, U., Reichling, J., Ruth, P., & Schulz, V. (Eds). (2014). *Hagerrom 2014. Hagers Enzyklopadie Der Arzneistoffe Und Drogen: Einzelplatzversion* (12th edn). Springer.

Božić, D., Skorić, M., Šiler, B., Živković, S., Dmitrović, S., Banjanac, T., & Mišić, D. (2015). Secoiridoid glycosides production in hairy roots of *Gentiana pneumonanthe* L.: Optimization of cytokinin concentration in the culture medium. *Bot. Serb.* 39: 79–86.

Chen, H., Liu, P., Yu, R., Mohammadtursun, N., Aikemu, A., & Yang, X. (2024). Swertiamarin ameliorates type 2 diabetes by activating ADRB3/UCP1 thermogenic signals in adipose tissue. *Phytomedicine 135*: 156190. https://doi.org/10.1016/j.phymed.2024.156190

Chilton, M.-D., Tepfer, D. A., Petit, A., David, C., Casse-Delbart, F., & Tempé, J. (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature 295*(5848): 432–434. https://doi.org/10.1038/295432a0

Cuena-Lombraña, A., Porceddu, M., Dettori, C. A., & Bacchetta, G. (2018). Discovering the type of seed dormancy and temperature requirements for seed germination of *Gentiana lutea* L. subsp. *lutea* (Gentianaceae). *J. Plant Ecol. 11*(2): 308–316. https://doi.org/10.1093/jpe/rtx003

Demirci, T., Albayrak, I., Deveci, H., Asci, Ö. A., & Baydar, N. G. (2024). Development of effective micropropagation protocols for endangered *Gentiana lutea* and *Gentiana boissieri* (endemic to Türkiye). *Isr. J. Plant Sci. 71*(3–4): 127–136. https://doi.org/10.1163/22238980-bja10108

Ehrendorfer, L. E., Niklfeld, H., Schröck, C., Stöhr, O., Gilli, C., Sonnleitner, M., Adler, W., Barta, T., Beiser, A., Berg, C., Bohner, A., Franz, W. R., Gottschlich, G., Griebl, N., Haug, G., Heber, G., Hehenberger, R., Hofbauer, M., Hohla, M., Zuna-Kratky, T. (2022). *Rote Liste der Farn- und Blütenpflanzen Österreichs*. Land Oberösterreich, Biologiezentrum der Oberösterr. Landesmuseen.

Espinosa-Leal, C. A., Puente-Garza, C. A., & García-Lara, S. (2018). *In vitro* plant tissue culture: Means for production of biological active compounds. *Planta 248*(1): 1–18. https://doi.org/10.1007/s00425-018-2910-1

Europäisches Arzneibuch. (2023). Deutscher Apotheker Verlag.

Falcone, G., Di Matteo, G., Mannina, L., Cellini, F., Menghini, L., Carradori, S., Al-Samydai, A., Chiavaroli, A., Di Simone, S. C., & Ferrante, C. (2025). Phytochemical profiling and anti-inflammatory activity of *Gentiana lutea* roots from Pollino National Park. *J. Ethnopharmacol. 353*: 120430. https://doi.org/10.1016/j.jep.2025.120430

Gamborg, O. L., Miller, R. A., & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res. 50*(1): 151–158. https://doi.org/10.1016/0014-4827(68)90403-5

Georgiev, M. I., Ludwig-Müller, J., Alipieva, K., & Lippert, A. (2011). Sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum* Griseb. For bioactive metabolite accumulation. *Plant Cell Rep. 30*(5): 859–866. https://doi.org/10.1007/s00299-010-0981-y

González-López, Ó., & Casquero, P. A. (2014). Effects of GA_3 Pregerminative Treatment on *Gentiana lutea* L. var. *Aurantiaca* Germination and Seedlings Morphology. *Sci. World J. 2014*: 1–6. https://doi.org/10.1155/2014/751279

Gutierrez-Valdes, N., Häkkinen, S. T., Lemasson, C., Guillet, M., Oksman-Caldentey, K.-M., Ritala, A., & Cardon, F. (2020). Hairy Root Cultures—A Versatile Tool With Multiple Applications. *Front. Plant Sci. 11*: 33. https://doi.org/10.3389/fpls.2020.00033

Hayta, S., Gurel, A., Akgun, I. H., Altan, F., Ganzera, M., Tanyolac, B., & Bedir, E. (2011). Induction of *Gentiana cruciata* hairy roots and their secondary metabolites. *Biologia 66*(4): 618–625. https://doi.org/10.2478/s11756-011-0076-4

Hwang, H.-H., Yu, M., & Lai, E.-M. (2017). *Agrobacterium* -Mediated Plant Transformation: Biology and Applications. *Arabidopsis Book 15*: e0186. https://doi.org/10.1199/tab.0186

International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2023). *ICH Q2(R2) Validation of analytical procedures* (Q2(R2)). International Council for Harmonisation. https://database.ich.org/sites/default/files/ICH_Q2%28R2%29_Guideline_2023_1130.pdf

Jelitto Staudensamen GmbH. (n.d.). *Aussaatanleitung*. Jelitto Staudensamen GmbH. Retrieved https://www.jelitto.com/out/media/aussaatanleitungen/de.pdf

Keil*, M., Härtle, B., Guillaume, A., & Psiorz, M. (2000). Production of Amarogentin in Root Cultures of *Swertia chirata*. *Planta Med. 66*(5): 452–457. https://doi.org/10.1055/s-2000-8579

Lacroix, B., & Citovsky, V. (2019). Pathways of DNA Transfer to Plants from *Agrobacterium tumefaciens* and Related Bacterial Species. *Annu. Rev. Phytopathol. 57*(1): 231–251. https://doi.org/10.1146/annurev-phyto-082718-100101

Leong, X. Y., Thanikachalam, P. V., Pandey, M., & Ramamurthy, S. (2016). A systematic review of the protective role of swertiamarin in cardiac and metabolic diseases. *Biomed. Pharmacother. 84*: 1051–1060. https://doi.org/10.1016/j.biopha.2016.10.044

Li, X., Yang, C., & Shen, H. (2019). Gentiopicroside exerts convincing antitumor effects in human ovarian carcinoma cells (SKOV3) by inducing cell cycle

arrest, mitochondrial mediated apoptosis and inhibition of cell migration. *JBUON* 24(2): 635–640.

Li, X., Zhang, Y., Jin, Q., Xia, K., Jiang, M., Cui, B., Wu, Y., Song, S., Lian, L., & Nan, J. (2018). Liver kinase B1/AMP-activated protein kinase-mediated regulation by gentiopicroside ameliorates P2X7 receptor-dependent alcoholic hepatosteatosis. *Br. J. Pharmacol. 175*(9): 1451–1470. https://doi.org/10.1111/bph.14145

Liu, C., Ahmad, N., Tao, Y., Hussain, H., Chang, Y., Umar, A. W., & Liu, X. (2025). Reprogramming Hairy Root Cultures: A Synthetic Biology Framework for Precision Metabolite Biosynthesis. *Plants 14*(13): 1928. https://doi.org/10.3390/plants14131928

Marković, T., Radanović, D., Nastasijević, B., Antić-Mladenović, S., Vasić, V., & Matković, A. (2019). Yield, quality and safety of yellow gentian roots produced under dry-farming conditions in various single basal fertilization and planting density models. *Ind. Crops Prod. 132*: 236–244. https://doi.org/10.1016/j.indcrop.2019.02.027

Mirzaee, F., Hosseini, A., Jouybari, H. B., Davoodi, A., & Azadbakht, M. (2017). Medicinal, biological and phytochemical properties of *Gentiana* species. *Tradit. Complement. Med 7*(4): 400–408. https://doi.org/10.1016/j.jtcme.2016.12.013

Momčilović, I., Grubišić, D., Kojić, M., & Nešković, M. (1997b). *Agrobacterium rhizogenes*-mediated transformation and plant regeneration of four *Gentiana* species. *Plant Cell Tissue Organ Cult. 50*(1): 1–6. https://doi.org/10.1023/A:1005880802231

Momčilović, I., Grubišić, D., & Nešković, M. (1997a). Micropropagation of four *Gentiana* species (*G. lutea*, *G. cruciata*, *G. purpurea* and *G. acaulis*). *Plant Cell Tissue Organ Cult.* 49: 141–144.

Moussous, A., Paris, C., Khelifi-Slaoui, M., Bekhouche, M., Zaoui, D., Rosloski, S. M., Makhzoum, A., Desobry, S., & Khelifi, L. (2018). Pseudomonas spp. Increases root biomass and tropane alkaloid yields in transgenic hairy roots of *Datura* spp. *In Vitro Cell. Dev. Biol. Plant 54*(1): 117–126. https://doi.org/10.1007/s11627-017-9862-1

Murthy, H. N., Lee, E. J., & Paek, K. Y. (2014). Production of secondary metabolites from cell and organ cultures. *In Vitro Cell. Dev. Biol. Plant 50*(1): 1–17.

Mustafa, A. M., Caprioli, G., Ricciutelli, M., Maggi, F., Marín, R., Vittori, S., & Sagratini, G. (2015). Comparative HPLC/ESI-MS and HPLC/DAD study of different populations of cultivated, wild and commercial *Gentiana lutea* L. *Food Chem. 174*: 426–433. https://doi.org/10.1016/j.foodchem.2014.11.089

Pan, Y., Zhao, Y., Zhang, J., Li, W., & Wang, Y. (2016). Phytochemistry and Pharmacological Activities of the Genus *Gentiana* (Gentianaceae). *Chem. Biodiversity 13*(2): 107–150. https://doi.org/10.1002/cbdv.201500333

Radanović, D., Marković, T., Aiello, N., & Fusani, P. (2014). Cultivation trials on *Gentiana lutea* L. in Southern and South-eastern Europe. *J. Appl. Res. Med. Aromat. Plants 1*(4): 113–122. https://doi.org/10.1016/j.jarmap.2014.10.004

Rahmat, E., & Kang, Y. (2019). Adventitious root culture for secondary metabolite production in medicinal plants: A Review. *J. Plant Biotechnol. 46*(3): 143–157. https://doi.org/10.5010/JPB.2019.46.3.143

Ramachandra Rao, S., & Ravishankar, G. A. (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances 20*(2): 101–153. https://doi.org/10.1016/S0734-9750(02)00007-1

Ren, G., Zhang, Q., Xia, P., Wang, J., Fang, P., Jin, X., Peng, X., Xu, Y., Zhang, J., & Zhao, L. (2023). Synthesis and Biological Evaluation of Gentiopicroside Derivatives as Novel Cyclooxygenase-2 Inhibitors with Anti-Inflammatory Activity. *Drug Des. Devel. Ther. 17*: 919–935. https://doi.org/10.2147/DDDT.S398861

Santos, P. A. G., Figueiredo, A. C., Oliveira, M. M., Barroso, J. G., Pedro, L. G., Deans, S. G., & Scheffer, J. J. C. (2005). Growth and essential oil composition of hairy root cultures of *Levisticum officinale* W.D.J. Koch (lovage). *Plant Sci. 168*(4): 1089–1096. https://doi.org/10.1016/j.plantsci.2004.12.009

Seitz, R., Lange, D., & Franz, C. (2005). Gelber Enzian. *Z. Phytother. 26*(3): 143–151. https://doi.org/10.1055/s-2005-872307

Shakeran, Z., Keyhanfar, M., & Ghanadian, M. (2017). Biotic elicitation for scopolamine production by hairy root cultures of *Datura metel*. *Mol. Biol. Res. Commun.* 6(4): 157–165. https://doi.org/10.22099/mbrc.2017.25776.1275

Sharma, M., Malim, F. M., Goswami, A., Sharma, N., Juvvalapalli, S. S., Chatterjee, S., Kate, A. S., & Khairnar, A. (2023). Neuroprotective Effect of Swertiamarin in a Rotenone Model of Parkinson's Disease: Role of Neuroinflammation and Alpha-Synuclein Accumulation. *ACS Pharmacol. Transl. Sci. 6*(1): 40–51. https://doi.org/10.1021/acsptsci.2c00120

Sobot, A. V., Savic, J., & Filipovic Trickovic, J. (2020). Toxicity assessment of *Gentiana lutea* L. root extract and its monoterpene compounds. *Indian J. Exp. Biol.* 58: 609–616. https://doi.org/10.56042/ijeb.v58i09.39874

Song, B., & Zhou, W. (2022). Amarogentin has protective effects against sepsis-induced brain injury via modulating the AMPK/SIRT1/NF-κB pathway. *Brain Res. Bull. 189*: 44–56. https://doi.org/10.1016/j.brainresbull.2022.08.018

Tabata, H. (2004). Paclitaxel Production by Plant-Cell-Culture Technology. In *Adv. Biochem. Eng. Biotechnol* 7: 1–23 Springer Berlin Heidelberg. https://doi.org/10.1007/b13538

Tan, Z., Wang, W., Peng, J., Zhou, Z., Pan, J., Peng, A., Cao, H., & Fan, W. (2022). Impact of Amarogentin on Gastric Carcinoma Cell Multiplication, Apoptosis and Migration via circKIF4A/miR-152-3p. *J. Immunol. Res. 2022*: 1–9. https://doi.org/10.1155/2022/2156204

Tiwari, R. K., Trivedi, M., Guang, Z. C., Guo, G.-Q., & Zheng, G.-C. (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*(2): 199–210. https://doi.org/10.1007/s00299-006-0236-0

Trick, H. N., & Finer, J. J. (1997). SAAT: sonication-assisted *Agrobacterium*-mediated transformation. *Transgenic Res.* 6(5): 329–336. https://doi.org/10.1023/a:1018470930944

Vinterhalter, B., Banjac, N., Vinterhalter, D., & Krstić-Milošević, D. (2021). Xanthones Production in *Gentiana dinarica* Beck Hairy Root Cultures Grown in Simple Bioreactors. *Plants 10*(8): 1610. https://doi.org/10.3390/plants10081610

Vinterhalter, B., Krstić-Milošević, D., Janković, T., Pljevljakušić, D., Ninković, S., Smigocki, A., & Vinterhalter, D. (2015). *Gentiana dinarica* Beck. Hairy root cultures and evaluation of factors affecting growth and xanthone production. *Plant Cell Tissue Organ Cult. 121*(3): 667–679. https://doi.org/10.1007/s11240-015-0737-z

Wang, T., Wu, S., Ibrahim, I. A. A., & Fan, L. (2023). Cardioprotective Role of Swertiamarin, a Plant Glycoside Against Experimentally Induced Myocardial Infarction via Antioxidant and Anti-inflammatory Functions. *Appl. Biochem. Biotechnol. 195*(9): 5394–5408. https://doi.org/10.1007/s12010-022-04094-1

Wawrosch, C., & Zotchev, S. B. (2021). Production of bioactive plant secondary metabolites through *in vitro* technologies—Status and outlook. *Appl. Microbiol. Biotechnol. 105*(18): 6649–6668. https://doi.org/10.1007/s00253-021-11539-w

Xu, Y., Li, Y., Maffucci, K., Huang, L., & Zeng, R. (2017a). Analytical Methods of Phytochemicals from the Genus *Gentiana*. *Molecules 22*(12): 2080. https://doi.org/10.3390/molecules22122080

Zhang, H. L., Xue, S. H., Pu, F., Tiwari, R. K., & Wang, X. Y. (2010). Establishment of hairy root lines and analysis of gentiopicroside in the medicinal plant *Gentiana macrophylla*. *Russ. J. Plant Physiol. 57*(1): 110–117. https://doi.org/10.1134/S1021443710010152

Zhu, Y., Zhu, X., Wen, Y., Wang, L., Wang, Y., Liao, C., Zhao, M., Li, T., Liu, D., Li, B., Zhu, T., & Wang, L. (2024). Plant hairy roots: Induction, applications, limitations and prospects. *Ind. Crops Prod. 219*: 119104. https://doi.org/10.1016/j.indcrop.2024.119104