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„Establishment of *in vitro* hairy root cultures of *Leontopodium nivale* subsp. *alpinum* and analysis of secondary metabolites"

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Abbreviations

HPLC	High performance liquid chromatography
LA	*Leontopodium (nivale* subsp.) *alpinum*
LB	Lysogeny broth
MS	Murashige and Skoog medium
OD	Optical density
PCR	Polymerase chain reaction
Ri plasmid, pRi	Root-inducing plasmid
RPM	Revolutions per minute
T-DNA	Transfer-DNA
YEB	Yeast extract beef medium

1. Abstract

Leontopodium nivale subsp. *alpinum*, commonly known as Edelweiss, is an alpine perennial plant that has been used for centuries to treat diseases like abdominal pain or disorders of the respiratory tract in traditional European folk medicine. In the past few decades, its bioactive compounds revealed interesting pharmacological properties such as antioxidative, anti-inflammatory and vaso- and cardioprotective effects. However, the commercial use of this plant is restricted to this date, due to low content of the compounds. The present study initially aimed to establish a plant biotechnological method using Edelweiss derived endophytes to enhance the production of bioactive secondary metabolites of *in vitro* hairy root cultures of Edelweiss. For this purpose, it was planned to initiate Edelweiss seed cultures and later induce hairy root formation by infecting young plantlets with the bacterium *Rhizobium rhizogenes* to generate the starting material for the subsequent experiments. Aqueous solutions of sodium hypochlorite, containing 2.8 to 7% active chlorine, were utilized for seed sterilization. In 30 sterilization attempts, an unusual high microbial load of the seed material was revealed, resulting in only 0 to 50% sterile seed cultures per batch. To obtain the necessary number of Edelweiss cultures for the infection experiments, sterile cultures were further propagated and supplemented by additional Edelweiss plantlets deriving from clonal lines that were initiated in 2020. Various strategies for plant infection were elaborated and applied using *Rhizobium rhizogenes* strains ATCC15834, A4, LBA9402 and 8691. In over 200 infection experiments with a co-cultivation period of 12 weeks, hairy root formation could not be successfully induced. Through extensive literature research it was attempted to point out possible causes and outline alternative strategies for further studies.

In order to acquire practical skills in phytochemical analysis, it was decided to investigate already established hairy root samples of *Atropa belladonna* L. regarding their content of secondary metabolites. The tropane alkaloids atropine and scopolamine were extracted and quantified by HPLC analysis using external standards. All ten measured samples exhibited an average content of 418.34 µg atropine and 151.53 µg scopolamine per gram drug sample with an average atropine:scopolamine ratio of 3.5.

Zusammenfassung

Leontopodium nivale subsp. *Alpinum,* auch bekannt als (Alpen-)Edelweiss, ist eine alpine krautige Pflanze, die in der traditionellen europäischen Volksmedizin seit Jahrhunderten zur Linderung von Krankheiten wie Bauchschmerzen oder Erkrankungen des Respirations-

trakts eingesetzt wird. In den letzten Jahrzehnten konnten interessante pharmakologische Eigenschaften der bioaktiven Inhaltsstoffe wie eine antioxidative, anti-inflammatorische und vaso- und kardioprotektive Wirkung nachgewiesen werden. Die kommerzielle Nutzung dieser Pflanze ist bis heute aufgrund des niedrigen Wirkstoffgehalts eingeschränkt. Die vorliegende Arbeit zielte anfänglich auf die Entwicklung einer pflanzenbiotechnologischen Methode ab, bei der Edelweiss-Endophyten verwendet werden, um die Produktion von bioaktiven Sekundärmetaboliten von *in vitro* Edelweiss-Kulturen zu erhöhen. Zu diesem Zweck sollten Samenkulturen des Edelweiss angelegt werden und anschließend junge Edelweiss-Pflanzen mit dem Bakterium *Rhizobium rhizogenes* infiziert werden, um die Bildung von "hairy roots" zu induzieren und das Ausgangsmaterial für Folgeversuche zu erhalten. Wässrige Natriumhypochlorit-Lösungen, die 2,8 – 7 % aktives Chlor enthielten, wurden zur Samensterilisation verwendet. In 30 Sterilisationsversuchen wurde eine ungewöhnlich hohe mikrobielle Belastung des Samenmaterials festgestellt, wodurch pro Ansatz nur 0 – 50 % der Samenkulturen steril waren. Um die notwendige Anzahl an Edelweiss-Kulturen für die Infektionsexperimente zu erreichen, wurden die sterilen Kulturen vermehrt and durch zusätzliche Edelweiss-Pflanzen, die aus einer Klonlinie aus dem Jahr 2020 stammten, ergänzt. Verschiedene Strategien zur Pflanzeninfektion wurden erarbeitet und angewandt, wobei die *Rhizobium rhizogenes* Stämme ATCC15834, A4, LBA9402 und 8691 verwendet wurden. In über 200 Infektionsversuchen konnte nach 12-wöchiger Co-Kultivierung keine hairy roots-Bildung induziert werden. Anhand umfangreicher Literaturrecherche wurde versucht, mögliche Ursachen aufzuzeigen und alternative Strategien für zukünftige Studien zusammenzufassen.

Um praktische Fähigkeiten in der phytochemischen Analyse zu erwerben, wurde entschieden, bereits vorhandene hairy roots-Proben von *Atropa belladonna* L. hinsichtlich des Gehalts an Sekundärmetaboliten zu vermessen. Die Tropan-Alkaloide Atropin und Scopolamin wurden extrahiert und anschließend mittels HPLC Analyse mit externem Standard quantifiziert. Alle zehn vermessenen Proben zeigten einen durchschnittlichen Gehalt von 418,34 µg Atropin und 151,53 µg Scopolamin pro Gramm Ausgangsdroge, mit einem durchschnittlichen Verhältnis von Atropin zu Scopolamin von 3,5.

2. Introduction

2.1. *Leontopodium nivale* subsp. *alpinum*

Leontopodium nivale (Ten.) Huet. ex Hand.-Mazz. and *alpinum* Cass., commonly known as (Alpine) Edelweiss, is a perennial herbal plant of the Asteraceae family (Safer et al., 2011). The distribution range of its subspecies, *Leontopodium nivale* subsp. *alpinum* (Cass.) Greuter, extends throughout the central European alpine mountain regions, mostly the European Alps, the Pyrenees and the Balkan mountain range (Tauchen and Kokoska, 2017). Due to its preference to thrive in direct sunlight and its small height of up to 20 cm, it is often found on rocky grassland on an average elevation of 2,000 m above sea level, where it inhabits base-rich bedrocks such as limestone (Ischer et al., 2014). Originally, the broad majority of

FIGURE 1 | Specimen of *Leontopodium nivale* subsp. *alpinum*.

(https://www.researchgate.net/figure/Flowering-individual-of-Edelweiss-Leontopodium-nivale-ssp-alpinum-Cass-Greuter_fig3_305361385. Retrieved on May 25, 2022).

the genus *Leontopodium* with 41 species (Schwaiger et al., 2006) is distributed from the mountainous regions of central Asia until the eastern parts of the continent, from where it most likely has spread to the European subcontinent during warmer periods of Pleistocene (Blöch et al., 2010). *Leontopodium nivale* subsp. *alpinum* is a rosette plant that possesses overwintering buds, so called hemicryptophytes (Keller and Vittoz, 2015), and the floral head is characterized by up to 10 small yellow capitula and several white petal-like bracts that are coated with small hair (Ischer et al., 2014) (figure 1). For its visual appearance and rarity of locations, the Edelweiss plant is considered as cultural heritage (Safer et al., 2011) and is therefore often praised for its legendary symbolic value and its healing effects as a medicinal

plant (Ischer et al., 2014). After decades of intensive wild collection, Edelweiss is now a protected species in many European countries (Pralea et al., 2021), but according to the IUCN Red List of Threatened Species, the populations of Edelweiss are considered as stable (Khela, 2013).

In European folk medicine, the traditional use of different plant parts and extracts of Edelweiss has been passed down the generations for centuries (Safer et al., 2011). There are records of herbal medicinal preparations against respiratory and digestive diseases treated with infusions, tinctures or powders as well as treatments of various cancers such as breast cancer (Pralea et al., 2021). The blossom was often utilized for tea preparation or boiled in milk (Speroni et al., 2006) or wine (Tauchen and Kokoska, 2017). Other indications included abdominal illnesses, angina pectoris, diarrhea, dysentery (Dobner et al., 2003) and also heart diseases, fever, rheumatic pain and tonsilitis (Tauchen and Kokoska, 2017).

2.2. Bioactive secondary metabolites of Edelweiss

Nowadays, active secondary metabolites of Edelweiss are being investigated in both academia and industry. Due to its popularity and well-documented traditional use for centuries, extracts and especially isolated secondary metabolites of Edelweiss are subjects of comprehensive research and development (Tauchen and Kokoska, 2017). The increasing demand for plant material by both the cosmetic industry and for scientific research necessitated the development of plant production technologies on a larger scale (Pralea et al., 2021). Large amounts of plant material are currently produced by field cultivation in Switzerland ensuring the supply for the commercial use without endangering wild plant populations of Edelweiss (Schwaiger et al., 2006). Aside from crop cultivation, alternative approaches such as plant cell, tissue and organ culture techniques will presumably facilitate the access to bioactive substances and whole extracts of the plant for the industrial use (Tauchen and Kokoska, 2017).

For its antioxidative properties, extracts of aerial parts of the plant are utilized in cosmetic products, such as sunscreen (Schwaiger et al., 2006) or in anti-aging preparations (Pralea et al., 2021). Aside from the cosmetic industry, various bioactive compounds were assessed and confirmed regarding their pharmacological properties and therefore lay the foundation for the development of new plant-derived drugs and clinical treatments for different human diseases (Tauchen and Kokoska, 2017). During more than 50 years of research, a wide variety of secondary plant metabolites have been identified in Edelweiss: phenolic acids,

lignans, flavonoids, sesquiterpenes, coumarins, benzofuran and -pyrane analoga, polyacetylenes, diterpene acids and so forth (Schwaiger et al., 2005; Tauchen and Kokoska, 2017; Canton et al., 2021). Many of these classes of secondary metabolites are considered as protective agents with antioxidative properties. They are benefical for the plant's protection, especially against intense sunlight exposure (Canton et al., 2021). Schwaiger et al. (2006) reported that the content of phenolic acids, more specifically leontopodic acid derivates, decreases from the top of the plant, to the stem leaves and to the bottom leaves in relation to the intensity of UV radiation exposure. The same pattern was observed for the amount of flavonoids and its derivates showing the highest content determined in the capitula. However, an equal distribution pattern was revealed for chlorogenic acid and 3,5-dicaffeoylquinic acid, most likely depending on the time of harvest of the utilized plant material (Schwaiger et al., 2006).

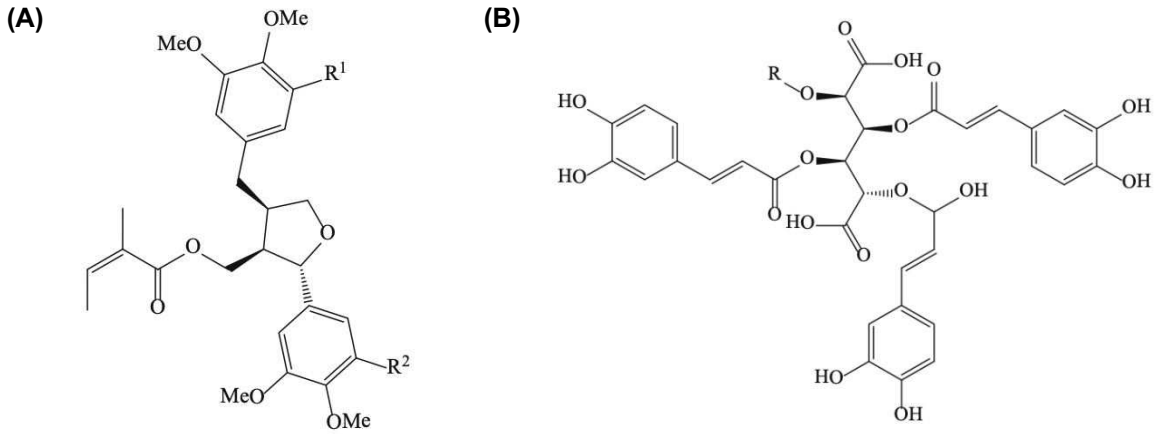


FIGURE 2 | Structures of selected constituents of *Leontopodium nivale* subsp. *alpinum*: **(A)** Leoligin (R^1 = H; R^2 = H) and 5-methoxy-leoligin (R^1 = H; R^2 = CH_3) **(B)** Leontopodic acid (R = $COCH_2COHCH_3$) and Leontopodic acid B (R = H)

(https://www.researchgate.net/publication/305361385_The_chemistry_and_pharmacology_of_Edelweiss_a_review. Retrieved on May 25, 2022).

Several interesting activities of both extracts as well as isolated bioactive secondary metabolites of Edelweiss have been reported. Some constituents from the roots of Edelweiss, among them one compound of the lignan class, revealed a strong inhibition of the leukotriene biosynthesis (*in vitro*). This anti-inflammatory effect was shown to be independent from the cyclooxygenase (COX)-1 and -2 inhibition (Schwaiger et al., 2004). Crude extracts also revealed anti-inflammatory activity in *in vivo* animal assays conducted by Dobner et al. (2004). A relatively well studied bioactive secondary metabolite of Edelweiss is leoligin (see figure 2 (A)) that belongs to the class of furan-type lignans. The highest concentration of leoligin was determined in the roots of Edelweiss showing anti-inflammatory activities. It was

revealed that analogues of leoligin exhibit a strong modulation of NF-κB activation which plays a leading role for the process of inflammation (Linder et al., 2021). A potential activity of leoligin was also revealed for the treatment of cardio vascular diseases showing cholesteryl ester transfer protein (CETP)-modulatory activity (Duwensee et al., 2011) and induction of cholesterol efflux in the THP-1 macrophages (Wang et al., 2016). In different human cell and animal models, leoligin additionally showed inhibition of TNF-α-mediated VCAM-1 expression which may significantly reduce the risk of hyperplasia of the tunica intima in veins (Reisinger et al., 2009). 5-Methoxy-leoligin (see figure 2 (A)) is a substituted structural analogue of leoligin that has recently been subject of comprehensive research for the first time. Linder et al. (2020) revealed, regarding its ability to induce cholesterol efflux in specific macrophages, targeted substitution of 5-methoxy-leoligin in 2-position showed identical properties compared to leoligin, whereas no activity was observed for the original substance 5-methoxy-leoligin (Linder et al., 2020). 5-Methoxy-leoligin also showed positive effects on both angiogenesis *ex vivo* and arteriogenesis in animal models (Messner et al., 2013). Together with the already reported antioxidative and DNA protective properties, 5-methoxy-leoligin revealed a similar or even twice as strong antioxidative effect in comparison to caffeic acid (Schwaiger et al., 2005). Leontopodic acid (see figure 2 (B)) and its derivates (e.g. leontopodic acid B) are a relatively sparsely investigated substance class. It is a fully substituted molecule of hexaric acid type primarily found in the floral head of Edelweiss. Additionally, it was shown that leontopodic acid might have cytoprotective traits against cytotoxicity induced by the mycotoxin deoxynivalenol (Costa et al., 2009).

The mentioned studies above clearly illustrate the yet unexploited potential of the pharmacological effects of secondary metabolites from Edelweiss. Until now, the biosynthetic pathways of these compounds are poorly understood (Linder et al., 2020) which might complicate the search for new bioactive agents and their (semi)synthetic production. Although the access to raw plant material is currently guaranteed via crop cultivation, new promising technologies, especially in the biotechnological field of plant cell, tissue and organ culture, may set the foundation for development of new plant-derived drugs to treat human diseases. A promising approach to increase the production of Edelweiss secondary metabolites was demonstrated by Wawrosch et al. (2014). In this study, the content of investigated constituents of *in vitro* hairy root cultures of Edelweiss were significantly increased by elicitation. A content of the bioactive compounds leoligin and 5-methoxy-leoligin at 0.0678% and 0.0372%, respectively, were found upon elicitation with 6% sucrose which is comparable to the lignan levels in usual roots of Edelweiss.

2.3. *In vitro* hairy root culture

Hairy root culture is a biotechnological approach that has been intensively studied for over three decades. This technique can be utilized to maximize the production of secondary metabolites by the plant tissue cultures (Gantait and Mukherjee, 2021). Hairy roots are originally described as the symptom of a plant disease, the hairy-root syndrome, caused by the soil-dwelling Gram-negative bacterium *Rhizobium rhizogenes*, formerly known as *Agrobacterium rhizogenes* (Veena and Taylor, 2007). By wounding plant tissue followed by infection with *R. rhizogenes*, copies of T-DNA (transfer-DNA) from the bacterial root-inducing (Ri) plasmid are transferred and integrated into the host plant cell's genome (Gantait and Mukherjee, 2021). The bacterium itself acts as a DNA delivery system to achieve transfer, integration and successful expression of the transgenic segments (Chandra, 2012). This leads to the production of opines that serve as an energy source for the bacteria (Chandra, 2012) and also stimulates the release of phytohormones, such as auxins and cytokinins, that result in the formation of adventitious roots (Gantait and Mukherjee, 2021). When cultivated *in vitro*, hairy roots frequently exhibit a rapid growth and increase in biomass. The supplementation with phytohormones to promote growth and proliferation can be omitted in most cases. Additionally, it is reported that the spectrum of secondary metabolites produced in hairy roots is in most cases identical to that of the plant's wild type roots (Veena and Taylor, 2007).

However, this biological system is limited facing difficulties on the molecular level such as random gene silencing and poor gene expression of T-DNA genes that result in a decrease of transformation efficiency (Gantait and Mukherjee, 2021). Other factors depend on the bacterial strain of *Rhizobium* and the density of the bacterial suspension used for infection (Georgiev et al., 2007). Multiple studies, such as Mahendran et al. (2022), Bhagat et al. (2019), and Saleh and Thuc (2009), revealed the beneficial use of exogenous acetosyringone applied during infection, to promote induction frequency of hairy roots. The plant normally releases endogenous acetosyringone resulting from mechanical damage of the plant tissue which is later detected by *Rhizobium* through chemotaxis (Baker et al., 2005). As a consequence, the activation of *vir* (virulence) genes, located on the Ri plasmid of the bacterium, is stimulated which enhances induction frequency (Mahendran et al., 2022). The application of exogenous acetosyringone is commonly used for *in vitro* hairy root induction.

3. Aim of this work

A previous study on *in vitro* hairy root cultures of *Leontopodium nivale* subsp. *alpinum*, conducted by Wawrosch et al. (2014), revealed a significant increase in the content of leoligin and 5-methoxy-leoligin after elicitation with 6% sucrose. Another strategy for the enhancement of secondary metabolites is the co-cultivation of hairy root cultures with bacteria acting as biotic elicitors.

The initial aim of this work was to obtain *in vitro* hairy root cultures of Edelweiss and to analyze the transgenic material regarding its content of secondary metabolites after the elicitation with Edelweiss-specific endophytes. Due to the fact, that the infection experiments to induce hairy root formation were not successful, it was necessary to acquire skills in phytochemical analysis by investigating already established hairy root samples of *Atropa belladonna* L.

4. Material and Methods

4.1. Plant material

Seeds of Edelweiss were purchased from Dehner Gartencenter Österreich GmbH & Co. KG, Pasching, Austria (2021A059, MHD 07/2023), Austrosaat AG, Vienna, Austria (MHD12/2024, Füllung 2022) and Jelitto Staudensamen GmbH, Schwarmstedt, Germany (Partie Nr. LA10080034031AA1AA02s).

In vitro cultures were obtained by propagating Edelweiss shoot cultures which had been established by Mag. Silvia Löbsch (Department of Pharmaceutical Sciences, Division of Pharmacognosy, University of Vienna, Austria). The seeds for these cultures had been purchased from Austrosaat AG, Vienna, Austria (MHD 12/2022, Füllung 2020) and the cultures have been initiated in June 2020.

4.2. Plant culture media

For seed germination as well as for shoot culture propagation, modified half-strength semi-solid MS medium (Murashige and Skoog, 1962), in this work referred as 0.5 MS medium, was used throughout all experiments. For media preparation, exactly measured aliquots of liquid stock solutions of macro-nutrients (KNO_3, NH_4NO_3, $CaCl_2$, KH_2PO_4, $MgSO_4$), $FeSO_4$, vitamins and micro-nutrients, were mixed with the necessary amount of distilled water. All stock solutions were stored at 4°C, except for the KNO_3 solution which was kept at room temperature. The 0.5 MS medium was subsequently enriched with organic compounds, namely 10 g/L of sucrose and 100 mg/L of myo-inositol (Merck KGaA, Darmstadt, Germany). The medium was fortified with 3 g/L of Gelrite™ (Carl Roth GmbH + Co KG, Karlsruhe, Germany) and finally adjusted to a pH-value of 5.7 ± 0.1. All chemicals were of consistent laboratory quality except for the sucrose that was of household quality. 40 mL of culture medium was filled up in 100 mL HIPP™ jars capped with Magenta™ B-caps. The medium was autoclaved at 121 °C for 20 minutes and stored at 4°C in the dark until use.

4.3. Plant culture conditions

Plant cultures were incubated in growth chambers at 25°C and 50% relative humidity under a 16 hours photoperiod with a light intensity of 40 $\mu M \cdot m^{-2} \cdot s^{-1}$.

4.4. *In vitro* cultures of *Leontopodium nivale subsp. alpinum*

4.4.1. Surface sterilization and aseptic germination of seeds

For surface sterilization, a small amount of Edelweiss seeds were centrically placed on a peace of water-permeable cloth and closed to a small bag with a rubber band. The formed bag should have a sufficiently large volume to allow the seeds to circulate freely to prevent the seeds from aggregating. To assess the efficiency of the sterilization method, various concentrations of an aqueous sodium hypochlorite solutions were tested. A concentrated aqueous sodium hypochlorite solution (W. Neuber's Enkel GmbH, Vienna, Austria; 14% active chlorine) was diluted with distilled water in a 50 mL Falcon tube to achieve a concentration of the sterilization solution between 20 to 50% (v/v; 2.8 to 7% active chlorine). 1-2 drops of Tween 20 (AppliChem GmbH, Darmstadt, Germany) per 50 mL were added as detergent and mixed well. The water-permeable cloth containing the Edelweiss seeds was subsequently submerged in the sterilization solution for an exposure time of 30-50 minutes, with frequent intermixing. After discarding of the sterilizing solution, the seeds were rinsed 4 times with sterile distilled water. For the first washing step, the falcon tube was filled up with autoclaved water, and after thorough mixing the water was immediately discarded. The procedure was repeated three more times, but the seeds were kept in the water for 10 minutes, respectively, before discarding. Subsequently, the surface sterilized Edelweiss seeds were transferred to 0.5 MS medium under sterile conditions. The cultures were then transferred to the growth chamber for seed germination and further development of Edelweiss shoots. In sum, 30 sterilization experiments were conducted between March and April 2022. One single batch included 4 to 12 culture vessels with approximately 15 seeds per vessel.

The efficiency of surface sterilization was evaluated throughout the sterilization treatments by taking samples of the utilized rubber bands and of the last washing water. These samples were transferred to 0.5 MS medium and incubated for several days.

The seed germination rate was evaluated, as well. Per batch, 3 culture vessels of each sterile or contaminated batches were analyzed and germination rate was determined by dividing the number of seeds that sprout by the total number of seeds per culture vessel, and then multiplying that number by 100.

4.4.2. Propagation of shoot cultures

The propagation of already established *in vitro* shoot cultures of Edelweiss was considered as a back-up strategy to reach the necessary amount of shoot cultures for the infection experiments. Existing shoot cultures, initiated in 2020, were propagated by transferring side shoots (see figure 3) of the mother plant to semi-solid 0.5 MS medium. The resulting plant clones were further cultivated and served as new mother plants for subculturing every 4-5 weeks.

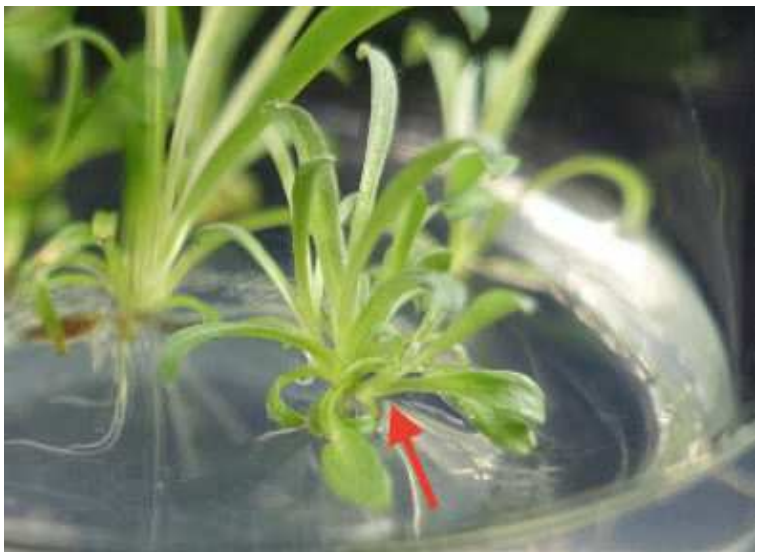


FIGURE 3 | Development of a side shoot (red arrow).

4.5. *Rhizobium rhizogenes*

In this Master's project, *Rhizobium rhizogenes* strains ATCC15834, A4, 8691 and LBA9402 were utilized. They were received as gifts from Dr. Szabolcs Béni (Department of Pharmacognosy, Semmelweis University, Budapest, Hungary) in June 2020 and from Dr. Aleksandra Królicka (Medical University of Gdansk, Gdansk, Poland) in July 2022. All strains were stored at -80°C in 20% glycerol until use.

R. rhizogenes strains ATCC15834, A4 and 8691 originally obtained from Hungary were taken over from Mag. Sophie Wöber (former Master student of Pharmacy at the Department of Pharmaceutical Sciences, Division of Pharmacognosy, University of Vienna, Austria) in

April 2022. The bacterial strains were plated on LB medium agar plates (Bertani, 1951) and stored at 4°C in the fridge.

Samples of *R. rhizogenes* strains ATCC15834, A4 and LBA9402 were also obtained from Poland. Bacteria were picked with an inoculation loop from the received Eppendorf vial and transferred to liquid YEB medium (Vervliet et al., 1975) and incubated on a incubator shaker overnight at 28°C and an agitation of 200 rpm. An inoculum of the bacterial suspension was then plated on YEB agar medium petri dishes and incubated overnight at 28°C. The plated *R. rhizogenes* strains were subsequently stored at room temperature in the dark until use.

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

Prior to the usage of the bacterial strains, the presence of the *rolB* gene was determined to give evidence of the proper functionality of the bacterium for transfection of T-DNA that leads to the formation of hairy roots.

In the first step, the bacterial plasmid DNA was isolated and purified using Wizard® Plus SV Minipreps DNA Purification System (Promega GmbH, Walldorf, Germany). For plasmid DNA extraction, a single colony of each bacterial strain was picked, transferred to 2 mL YEB medium and incubated overnight on a incubator shaker at 28°C and 200 rpm. The next day, 1.5 mL of the bacterial overnight suspension was transferred to a 1.5 mL Eppendorf vial and pelleted by centrifugation. Extraction protocol was followed and the isolated plasmid DNA was bound to the spin column of Wizard® purification system. After several washing steps, purified plasmid DNA was eluted with 100 µL nuclease-free water by centrifugation. The eluate of the bacterial plasmid DNA was collected in a 1.5 mL Eppendorf vial and stored at -20°C in a freezer until use.

To verify the successful extraction of plasmid DNA, 3-5 µL of the undiluted plasmid DNA extract was mixed up with the necessary amount of gel loading dye blue (6X) (New England Biolabs, Ipswich, United Kingdom) to a sample volume of 20 µL and loaded on a 0.8% TBE agarose gel supplemented with 50 µL/L GelRed® nucleic acid gel stain, 10,000X in DMSO (Biotium, Inc., Freemont, United States). The gel was run for 50 minutes at 100V in a horizontal gel electrophoresis chamber of Bio-Rad Laboratories GesmbH (Vienna, Austria) and later visualized with the ChemiDoc imaging system (Bio-Rad Laboratories GesmbH, Vienna, Austria).

Amplification was performed by PCR using *rolB* gene-specific oligonucleotide primers. The forward primer 5'-TGGATCCCAAATTGCTATTCACGA-3' and reverse primer 5'-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3' are designed to amplify the 0,779 kbp fragment of the *rolB* gene were adopted from Moussous et al. (2018) and Shakeran et al. (2017), and purchased from Eurofins Genomics Germany GmbH (Ebersberg, Germany). Q5 High Fidelity 2X Master Mix from New England Biolabs (Ipswich, United Kingdom) was used for amplification and the modified PCR protocols of MSc. Dr. Olha Schneider and Dr. Jaime Felipe Guerrero Garzón (both Department of Pharmaceutical Sciences, Division of Pharmacognosy, University of Vienna, Austria) were followed. Touchdown PCR was employed using the following PCR program: 1 cycle at 98°C for 10 min, 13 cycles at 98°C for 10 sec, 70-58°C for 30 sec *(rolB* primer annealing temperature was reduced at a rate of 1°C per cycle), 72°C for 30 sec, 27 cycles at 98°C for 10 sec, 57°C for 30 sec, 72°C for 30 sec and 1 cycle at 72°C for 10 min. The PCR premix contained 12.5 µL of Q5 High Fidelity 2X Master Mix, 1.25 µL of each oligonucleotide primers (diluted 1:10) and a certain volume of plasmid DNA sample of the respective *R. rhizogenes* strains filled up to 25 µL with autoclaved water. Various amounts of 1, 2 and 3 µL of plasmid DNA sample and two dilutions of 1:10 and 1:100 were tested to assess the appropriate intensity of the later gel bands. Different thermocycling conditions in multiple runs were employed to identify the optimal parameters.

To visualize the amplified PCR products, gel electrophoresis was performed in a 0.8% TBE agarose gel supplemented with 50 µL/L GelRed, as used previously for the extraction control. The gel was loaded with 1, 2 and 3 µL of the PCR products that was mixed with the necessary amount of gel loading dye blue (6X) (diluted 1:10) to a sample volume of 10 µL. The gel was run for ca 45 minutes at 100V for sufficient separation of the PCR fragments and the bands were later visualized on the ChemiDoc Imager.

4.7. Growth curves of the bacterial strains

Ahead of the infection experiments, each *R. rhizogenes* strain has been analyzed regarding its growth behavior by optical density (OD). For the determination of the bacterial growth, one single colony of each strain was picked and incubated in 2 mL of YEB on an incubator shaker at 28°C and 200 rpm for 24 hours to obtain seed cultures. A specific aliquot of the respective bacterial suspension varying from 1 to 20 µL was then transferred to a 100 mL Erlenmeyer flask with 20 mL YEB medium and incubated on the incubator shaker overnight. For the blank sample, 2 mL of YEB medium were transferred to a culture tube and incubated

overnight, as well. The following day, samples were drawn every 2 hours and optical density was measured with a UV-visible spectrophotometer (Evolution™ 260 Bio, Thermo Fischer Scientific Inc., Waltham, United States) against the blank YEB control at a wave length of 600 nm. Measurements for the growth curve terminated when an OD_{600} > 1.2 was obtained.

As described for example by Saleh and Thuc (2009), Muthusamy and Girija (2020) or Mahendran et al. (2022), transformation with different *R. rhizogenes* strains was most efficient with an OD_{600} of 0.6-0.8 which was adopted for the infection experiments of this work.

4.8. Infection experiments for hairy root induction

The aim of the experiments was to infect plant tissue with *R. rhizogenes* by wounding leaves of the Edelweiss plantlets. For preparation, two days prior to the experiment, 2 mL YEB medium in a culture tube were inoculated with one single colony of the respective *R. rhizogenes* strain and incubated overnight on an incubator shaker at 28°C and 200 rpm to obtain bacterial suspensions. The following day, a specific aliquot of overnight suspension was then transferred to 20 mL YEB medium in an Erlenmeyer flask and incubated on an incubator shaker at 28°C and 200 rpm. The specific aliquot and the duration of incubation depended on the bacterial growth of the respective *R. rhizogenes* strain (see chapter 5.3.2.). Each bacterial strain was used to infect 25-35 plantlets of Edelweiss.

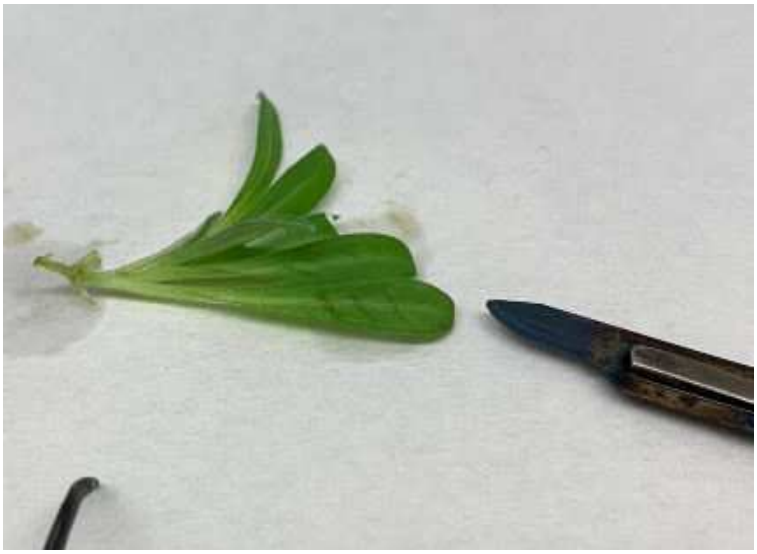


FIGURE 4 | Infection of Edelweiss plantlet by cutting wounds with a scalpel dipped in bacterial suspension.

Prior to the experiments, 192 µL of a filter-sterilized aqueous solution of acetosyringone (102.35 mg/100 mL; Sigma-Aldrich, subsidiary of Merck KGaA, Burlington, United States) were added to the bacterial suspension to achieve a final concentration of 50 µM (0.1962 mg acetosyringone per 20 mL bacterial suspension). The Edelweiss plantlets were placed on sterile paper. Roots, redundant leaves and side shoots were removed. The infection was performed by carefully cutting wounds into the main leaf vein with a scalpel that was previously dipped into bacterial suspension (Santos et al., 2005). Alternatively, some experiments were conducted by puncturing the leaves with a needle that was dipped into bacterial suspension. An illustration of the infection technique is depicted in figure 4. Afterwards, the plantlets were transferred to semi-solid 0.5 MS medium and further cultivated under usual culture conditions.

A second infection strategy was tested using excised leaves (Saleh and Thuc, 2009; Mahendran et al., 2022) that were previously isolated from Edelweiss plantlets. Leaves were cut into ca 5 x 5 mm explants and placed on a wetted sterile filter paper in a petri dish to avoid dehydration. Leaf explants were then submerged for 30 minutes in 20 mL bacterial suspension, as depicted in figure 5 (A). The vessel was agitated from time to time. Afterwards, the leaves were transferred to fresh sterile filter paper to remove excess bacterial suspension and ca 8-10 leaf explants were placed on semi-solid 0.5 MS medium. A piece of filter paper was used to cover up the explants in order to prevent withering (see figure 5 (B)). Half of the cultures were subsequently incubated under a 16 hours daylight rhythm, whereas the other half was kept in the dark. Both batches were co-cultivated for 3 or 5 days, respectively.

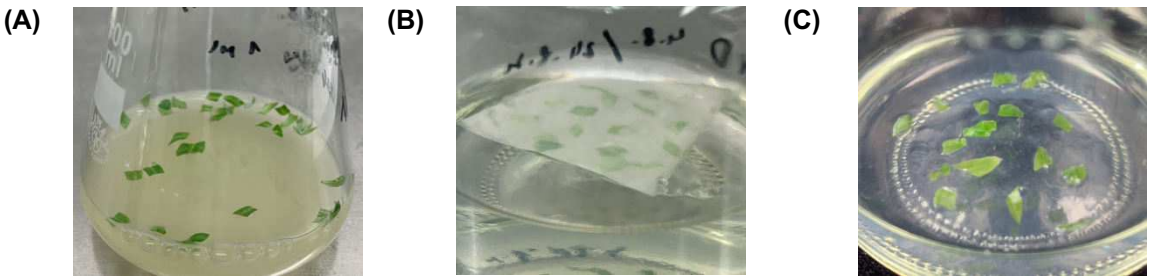


FIGURE 5 | Infection experiments with excised leaf explants of Edelweiss. **(A)** Explants were submerged in bacterial suspension for 30 minutes. **(B)** Covered with a peace of sterile filter paper, explants were incubated for co-cultivation. **(C)** After several washing steps, explants were placed on semi-solid 0.5 MS medium supplemented with 500 mg/L cefotaxime.

After this co-cultivation, the leaf explants were transferred to a 100 mL Erlenmeyer flask containing autoclaved water to rinse off leftover bacterial cells. The washing step was repeated three times, where the last washing solution was supplemented with 500 mg/L cefotaxime sodium salt (TCI Deutschland GmbH, Eschborn, Germany). The leaf explants were finally blotted dry on sterile filter paper and placed on semi-solid MS medium supplemented with 500 mg/L cefotaxime (see figure 5 (C)). The cultures were further cultivated under the usual culture conditions.

4.9. High Performance Liquid Chromatography (HPLC)

Lyophilized hairy root samples of *Atropa belladonna* L. were used to acquire skills and practice in quantifying plant secondary metabolites, namely tropane alkaloids such as atropine and scopolamine. High performance liquid chromatography (HPLC) was chosen as analytical method, 10 hairy root samples were provided from the previous Master's project of Mag. Sophie Wöber (2022).

4.9.1. Sample preparation

For sample preparation, the tropane alkaloid extraction protocol by Kamada et al. (1986) was followed. Initially, the dried samples were pulverized in a mortar. For extraction, 10 mL of a mixture of chloroform, methanol and ammonium hydroxide (15:5:1) was added to 100 mg dry sample and the suspension was sonicated for 10 mins. After sonification, the sample was kept at room temperature for 1 hour. The suspension was then filtrated and the residue was rinsed twice with 1 mL of chloroform. The collected filtrates were evaporated to dryness by using an rotavapor and the residue was redissolved with 5 mL of chloroform and 2 mL of 1N sulfuric acid and thoroughly mixed. The solution was then transferred to a 25 mL separating funnel where the chloroform phase was removed. The H_2SO_4 phase was alkalized to pH 10 in an ice bath by using ammonium hydroxide and subsequently extracted once with 2 mL and twice with 1 mL of chloroform. Before evaporating to dryness, the pooled extracts were dried with anhydrous sodium sulfate. The samples were stored at -20°C in a freezer until use. 10 minutes before analysis, the residue was redissolved in 1.00 mL of methanol (≥ 99.9%, Avantor Performance Materials Poland S.A., Gliwice, Poland) and centrifuged at 15,000 rpm for 10 min. All solvents were of HPLC grade.

4.9.2. HPLC method and conditions

Quantification of atropine and scopolamine was performed on a HPLC device from Shimadzu Corporation (Kyoto, Japan). HPLC equipment included a DGU-20A5R degrassing unit, a LC-20AT pump, a SIL-20AT HT autosampler, a CTO-20AC column oven, a SPD-M20A diode array detector and a CBM-20A communication module. A Luna® C18(2) column with a dimension of 250 x 4.6 mm and a particle diameter of 5 µm (Phenomenex Ltd., Aschaffenburg, Germany) was utilized.

A gradient elution described by Salem (2018) was applied with an aqueous solution of 0.1% formic acid (Merck KGaA, Darmstadt, Germany) as solvent A and acetonitrile (VWR International, Vienna, Austria) as solvent B. Table 1 depicts the HPLC gradient elution parameters. Column oven temperature was set to 25 °C, and the injection volume was 10 µL. Every sample was analyzed in triplicate.

TABLE 1 | Applied HPLC gradient elution parameters (Salem, 2018).

Time (min)	Solvent A (vol%)	Solvent B (vol%)
0.00	95	5
17.00	35	65
18.00	5	95
23.00	5	95
24.00	95	5
34.00	95	5

4.9.3. Alkaloid identification and quantification parameters

Identification and quantification was assessed using external standards of atropine and scopolamine. To illustrate the linear correlation between peak area and injection volume, respective calibration curves of atropine and scopolamine were generated. For this purpose, stock solutions of atropine and scopolamine hydrobromide trihydrate (TCI Deutschland GmbH, Eschborn, Germany) in methanol at concentrations of 1046.67 µg/mL and 1086.67 µg/mL, respectively, were prepared. Due to the fact that scopolamine hydrobromide trihydrate (384.26 g/mol) was used, a correction factor of 0.789 was applied to obtain the content of the alkaloid base (303.35 g/mol). By serial dilution, the stock solutions were diluted to six concentrations (261.67, 104.67, 52.33, 26.17, 5.23 and 2.62 µg/mL for atropine and 271.67,

108.67, 54.33, 27.17, 5.43, 2.72 µg/mL for scopolamine hydrobromide trihydrate). Dilutions were measured in triplicate with an injection volume of each 10 µL and the mean peak areas were plotted in a diagram against the corresponding concentration by using Microsoft Excel. The resulting trend line and coefficient of determination was used to estimate the correlation between peak area and injection volume and therefore evaluate the suitability of the HPLC method.

4.9.4. Peak evaluation and determination of content

HPLC data were analyzed with the software LabSolutions™ (Shimadzu Corporation, Kyoto, Japan). For peak identification of atropine and scopolamine, the retention time of the respective external standards were compared with the chromatogram of the hairy root samples. Peak integration was performed manually and the following equation, where "g" is the weighed portion and "analyte" is the alkaloid in the sample, was employed to calculate alkaloid content:

$$\%\ analyte = \frac{g(standard)\ *\ peak\ area(analyte)\ *\ 100}{peak\ area(standard)\ *\ g(sample)}$$

Due to the low content, the result was multiplied with the factor 10,000 and thus quantified as µg alkaloid per g hairy root material. The standard deviation was determined with MS Excel.

5. Results

5.1. Surface sterilization of seeds of *Leontopodium nivale subsp. alpinum*

To obtain *in vitro* cultures of Edelweiss, surface sterilization and subsequent aseptic germination of seeds was the strategy of first choice. Seeds were sterilized by using different concentrations between 20 to 50% (v/v) of an aqueous sodium hypochlorite solution and multiple washing steps with sterile water, as described in chapter 4.4.1. In a total of 30 experiments, it was attempted to determine the optimal sterilization parameters by varying the concentrations of the sterilization solutions and the respective incubation time during which the seeds were treated. After sterilization, approximately 15 seeds per culture vessel were placed on semi-solid 0.5 MS medium and incubated at 25°C under a 16 h light regime. One experiment hereby included 4-12 individual culture vessels. Throughout all experiments, an unusually high occurrence of microbial contaminations was observed and evaluated (see chapter 4.4.1.).

(A) **(B)**

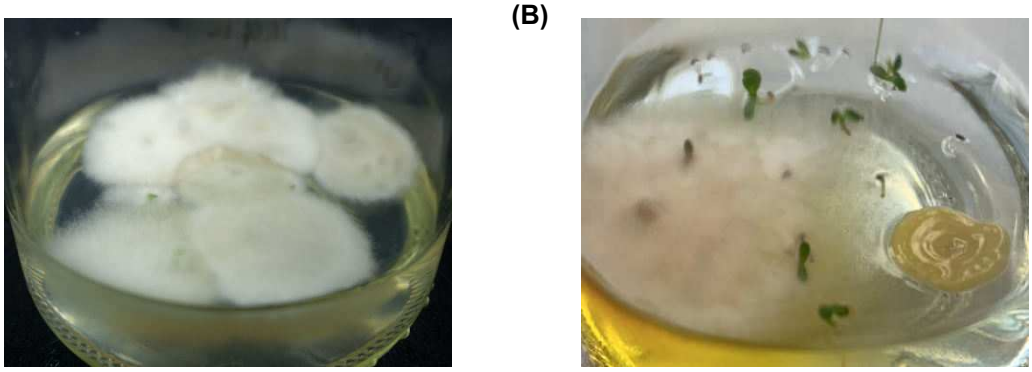


FIGURE 6 | Culture vessels showing microbial contaminations. **(A)** Multiple fungal contaminations 7 days after sterilization. **(B)** Fungal contamination with adjacent bacterial contamination.

Edelweiss seeds were initially purchased from Dehner Gartencenter Österreich GmbH & Co. KG (Pasching, Austria). However, only from 6 out of 12 seed batches from Dehner GmbH sterile cultures were obtained, with a relative frequency per batch ranging between 12.5 and 50.0%. First signs of microbial contaminations were evident ca 4-5 days after sterilization, showing most frequently initial growth of fungi, and occasional bacterial growth in less than 8% of all contaminated cultures after one week. It is important to note that the contaminations appeared to originate directly from the seed, starting simultaneously from multiple seeds and spreading quickly over the whole vessel. Typical contaminations are

illustrated in figure 6. After experiencing a high microbial contamination rate with the seeds from Dehner GmbH, it was decided to purchase additional seed material from Austrosaat AG (Vienna, Austria) and Jelitto Staudensamen GmbH (Schwarmstedt, Germany).

In total 183 cultures from Edelweiss seeds were initiated: 69 cultures from Dehner GmbH, 54 cultures from Austrosaat AG and 60 cultures from Jelitto GmbH seeds. 91.3% of all seed cultures turned out to be contaminated, with only 16 cultures being free of contamination. The respective contamination rate for each commercial seed source is shown in figure 7. Seeds from Dehner GmbH and Austrosaat AG showed a similar contamination rate of 88.4% and 85.2%, respectively. However, with the seeds purchased from Jelitto GmbH, no sterile seed culture at all could be obtained.

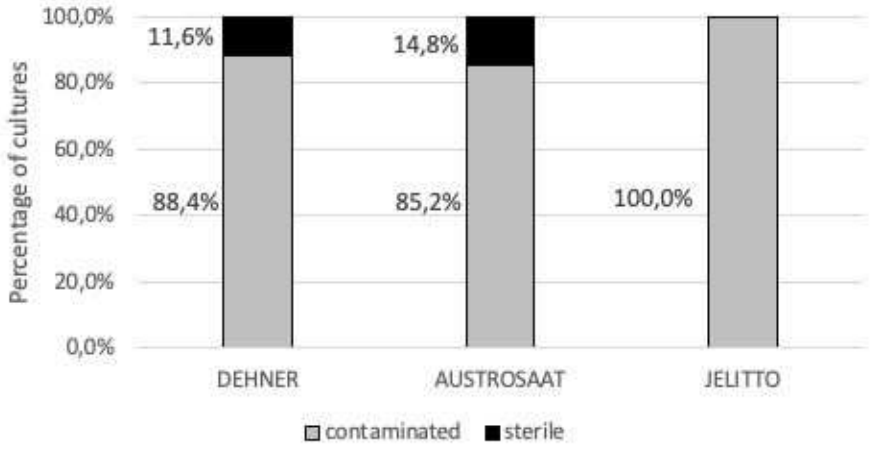


FIGURE 7 | Contamination rate depending on commercial source. Seed cultures from Jelitto GmbH showed a contamination rate of 100%.

Concerning the parameters of the surface sterilization treatments, the concentration of sodium hypochlorite and the duration of the treatment were adapted after each experiment. Table 2 shows the respective sterilization parameters and the percentage of sterile seed cultures that were observed throughout the experiments. With seeds from Dehner GmbH a minimum incubation time of 40 minutes with 40 or 50% NaOCl was necessary to obtain sterile seed cultures of Edelweiss, as depicted in figure 8. Sterilization efficiency was found to range between 12.5 and 50.0%. It seems that the concentration of sodium hypochlorite was not the pivotal parameter, due to the fact that for LA20 Dehner a concentration of 35% led to a sterilization efficiency of 50.0%, whereas a higher concentrated solution of 40% for

TABLE 2 | Percentage of sterile cultures per batch depending on commercial source, concentration of sodium hypochlorite solution and incubation time of the sterilization treatment.

Batch number	Concentration of NaOCl solution	Duration of treatment (min)	Percentage of sterile cultures per batch
LA1 Dehner	25%	40	0%
LA2 Dehner	30%	40	0%
LA3 Dehner	35%	40	0%
LA4 Dehner	40%	30	0%
LA5 Dehner	40%	40	12,50%
LA6 Dehner	45%	40	20,00%
LA7 Dehner	50%	30	0%
LA8 Dehner	50%	40	20,00%
LA9 Dehner	50%	45	33,33%
LA10 Dehner	50%	50	0%
LA19 Dehner	30%	40	25,00%
LA20 Dehner	35%	40	50,00%
LA11 Austrosaat	20%	30	14,29%
LA12 Austrosaat	25%	30	0%
LA13 Austrosaat	20%	40	16,67%
LA14 Austrosaat	25%	40	16,67%
LA15 Austrosaat	30%	30	0%
LA16 Austrosaat	40%	30	40,00%
LA17 Austrosaat	30%	40	20,00%
LA18 Austrosaat	40%	40	20,00%
LA21 Austrosaat	30%	40	25,00%
LA22 Austrosaat	35%	40	0%
LA23 Jelitto	20%	40	0%
LA24 Jelitto	25%	40	0%
LA25 Jelitto	20%	30	0%
LA26 Jelitto	25%	30	0%
LA27 Jelitto	40%	40	0%
LA28 Jelitto	45%	40	0%
LA29 Jelitto	40%	40	0%
LA30 Jelitto	45%	40	0%

LA5 Dehner resulted in only 12.5% sterile seed cultures. Presumably, the present inconsistency is caused by a lower infestation of LA20 Dehner with microorganisms located below the seed surface compared to LA5 Dehner.

Another aspect which supports this hypothesis is the fact that the highest utilized concentration (50%) with the longest incubation time (50 minutes), used for batch LA10, did not

lead to any sterile cultures. For the experiments with seeds from Austrosaat AG, lower concentrations of the sterilization solution and shorter incubation time tended to be more effective to achieve sterile seed cultures which might also be traced back to an unequal microbial load of the batches, as described in the previous paragraph. A concentration of at least 20% and 30 minutes of incubation time were necessary to accomplish seed surface sterility. Sterilization efficiency of the seeds purchased from Austrosaat AG was between 14.3 to 40.0%. A total of 8 sterilization experiments were also conducted with seeds from Jelitto GmbH, but it was not possible to obtain any sterile seed cultures even after a 40 min treatment with 45% NaOCl solution.

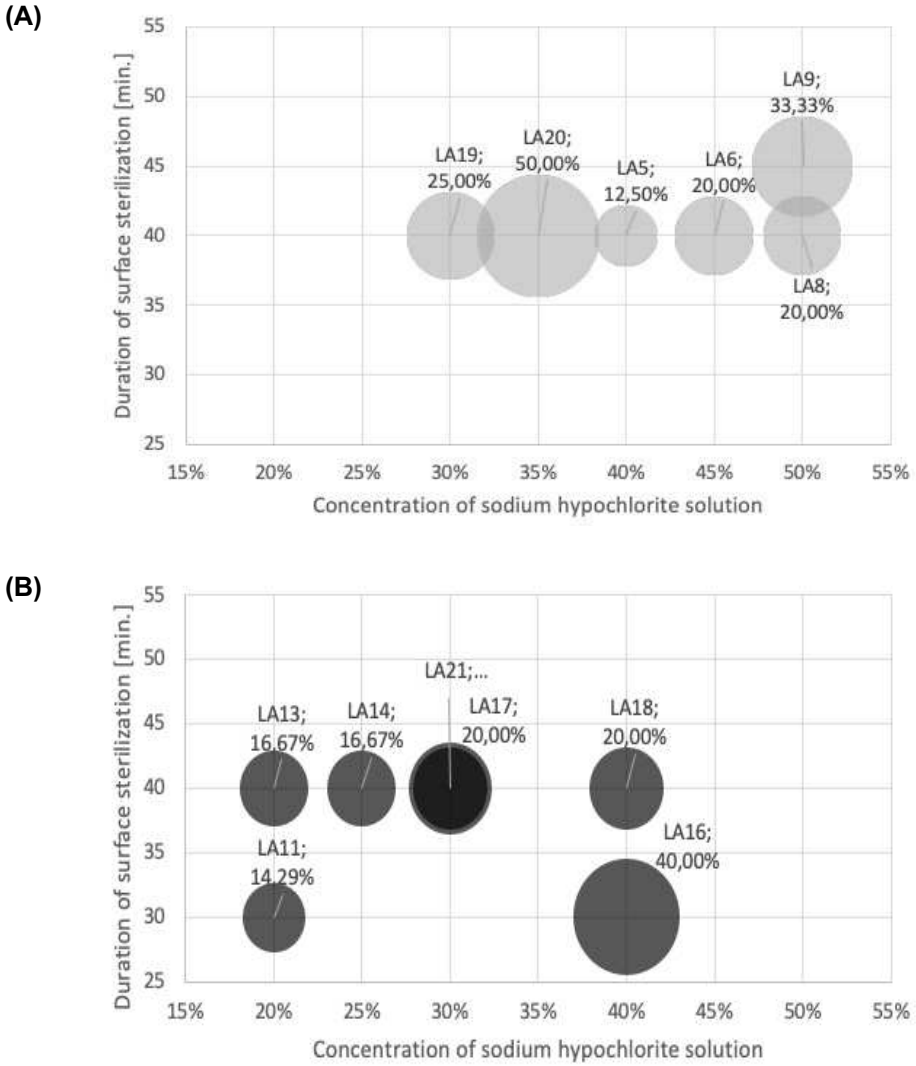


FIGURE 8 | Influence of sterilization conditions on sterility of seed cultures from **(A)** Dehner GmbH and **(B)** Austrosaat AG.

To directly assess the efficiency of surface sterilization, several samples of water that was used for the final washing step were inoculated on semi-solid 0.5 MS medium to confirm surface sterility of the treated seeds. In addition, samples of rubber bands that were used in the sterilization treatment (see chapter 4.4.1.) were placed on medium and incubated, as well. One week after initiation, no signs of contamination were evident, and these sterility tests were terminated after 10 days. Pictures of the experiments are presented in figure 9. These results confirmed the efficiency of the sterilization treatment regarding the elimination of microorganisms on the seed surface.

(A)

(B)

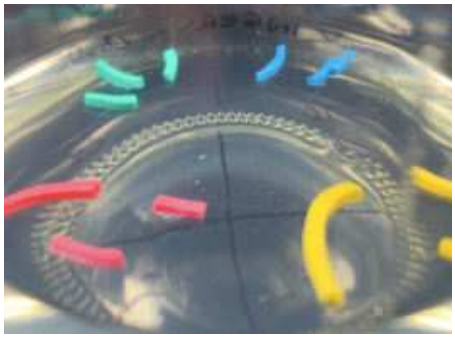


FIGURE 9 | Evaluation of surface sterilization efficiency by incubating samples of washing water **(A)** and rubber bands **(B)** that were used in the sterilization treatment. Both approaches did not show any signs of microbial contamination.

5.2. Germination of Edelweiss seeds

Within one week after sterilization, germination of the Edelweiss seeds was observed. For each sterilization batch (except the contaminated batches), germination rate was determined on the basis of 3 culture vessels. The germination rate was determined for 22 batches and the results are presented in figure 10 (A). Initially, the germination rate of the batches LA1, LA3 and LA4 were not further assessed and therefore excluded.

Germination was observed in every seed culture that was analyzed, regardless of whether the culture was sterile or contaminated. Seeds of Dehner GmbH and Jelitto GmbH showed higher germination rates as compared to seeds of Austrosaat AG. This is evident in figure 10 (B) showing almost identical germination rates of 70.0% and 73.2%, respectively, for the contaminated cultures of Dehner GmbH and Jelitto GmbH. Both sterile and contaminated cultures of Dehner GmbH seeds showed a respective 1.5 fold higher germination rate in

comparison to Austrosaat AG seeds with a rate of 48.4% for sterile and 46.5% for contaminated cultures. The present data suggests that, in general, the germination of Edelweiss seeds was not largely affected by the occurrence of microbial contamination. This can also be observed in figure 11 showing germinated seeds in both sterile (A) and contaminated (B) culture vessels.

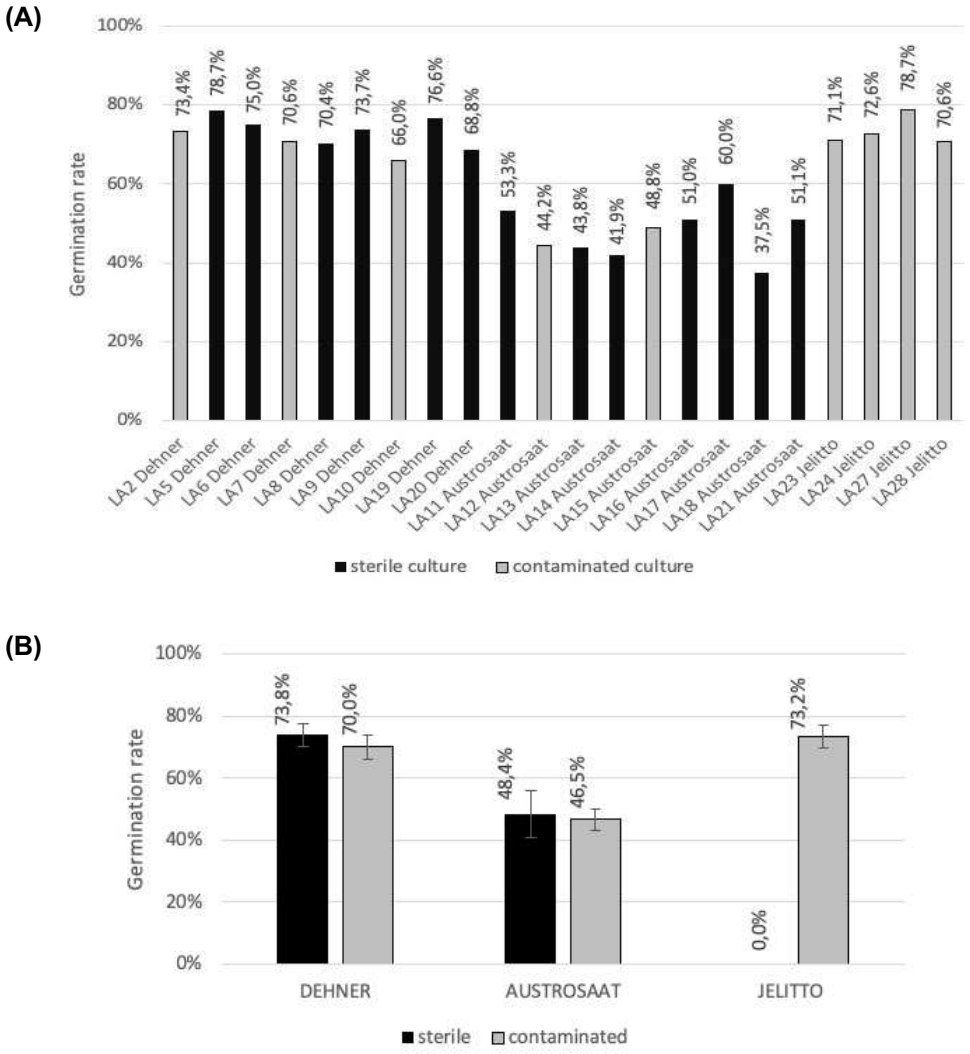


FIGURE 10 | Germination rate of Edelweiss seeds (mean values of 3 cultures). **(A)** Overview illustrating the germination rate per corresponding sterile or contaminated batch. **(B)** Average germination rate depending on commercial source with standard deviation.

(A) **(B)**

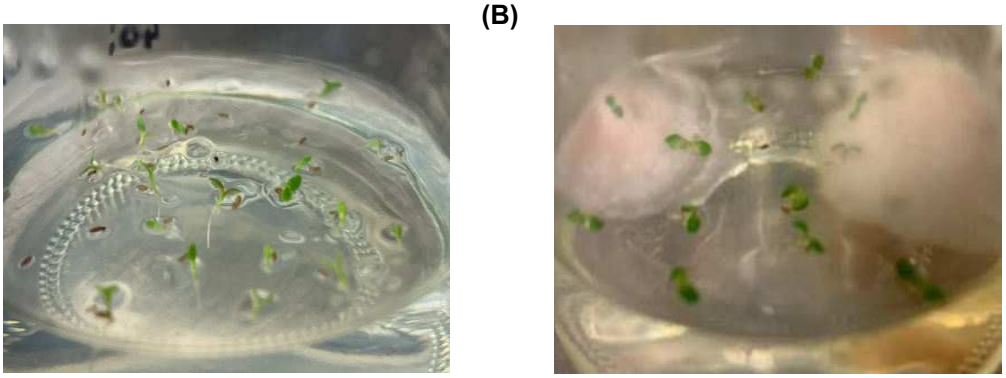


FIGURE 11 | *In vitro* germination of Edelweiss seeds. Germinated seeds of Dehner GmbH in a **(A)** sterile culture vessel and in a **(B)** contaminated culture vessel.

5.3. Establishment of *in vitro* hairy root cultures of *Leontopodium nivale subsp. alpinum*

5.3.1. Detection of *rolB* gene sequence in bacterial strains of *Rhizobium rhizogenes*

The utilized bacterial strains of *R. rhizogenes* were tested regarding the presence of the *rolB* gene to validate the capability of T-DNA transfer during the infection experiments (Gantait and Mukherjee, 2021). As mentioned in chapter 4.5. on page 16, the bacterial strains ATCC15834, A4 and LBA9402 obtained from Poland and ATCC15834, A4 and 8691 received from Hungary were utilized for infection of Edelweiss plantlets. For validation, plasmid DNA was extracted from overnight cultures in a first step and preliminary analyzed by gel electrophoresis for successful extraction (figure 12). Distinct plasmid bands for all bacterial strains could be detected at ca 10.0 kilobases. The presence of *rolB* gene of *R. rhizogenes* strain A4 from Hungary was successfully detected by Wöber (2022) and therefore excluded from this work.

Subsequently, the samples of isolated plasmid DNA were prepared for PCR (see chapter 4.6.) using *rolB*-specific oligonucleotide primers to amplify the *rolB* gene sequence of each bacterial strain. Due to absence of expected bands in preliminary experiments of this work, primer annealing temperature was set to touch down for 1°C per cycle reaching from 70°C to 57°C per cycle (see chapter 4.6.). Therefore, the possibility of an unsuitable primer annealing temperature when using a consistent temperature throughout the cycles, could be

ruled out. This strategy led in turn to the detection of previously unsuccessfully amplified *rolB* gene sequences.

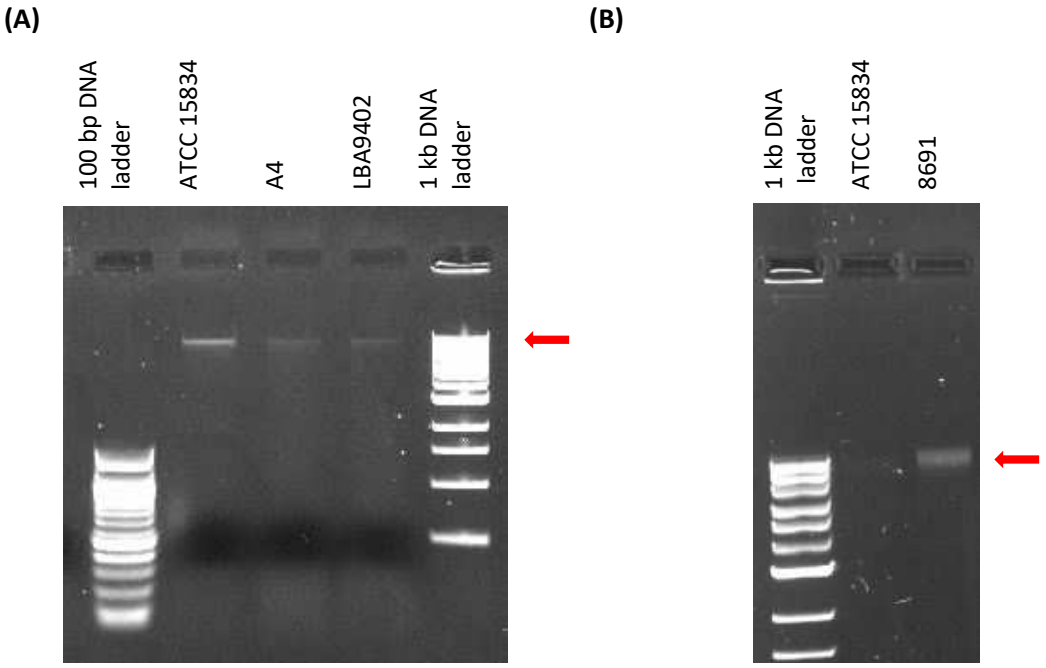


FIGURE 12 | Confirmation of successful plasmid DNA extraction of used *R. rhizogenes* strains by gel electrophoresis. Intensity of the bands might result from the different density of the respective bacterial culture that was used for analysis. **(A)** R. rhizogenes strains ATCC15834, A4 and LBA9402 obtained from Poland exhibited respective bands at ca 10.0 kilobases (arrow). **(B)** R. rhizogenes strains ATCC15834 and 8691 obtained from Hungary showed respective bands (arrow), as well.

PCR products were subsequently loaded on a gel and separated by electrophoresis for visualization and verification of the amplified *rolB* gene sequence. As depicted in figure 13, the presence of *rolB* gene could be unequivocally verified for the bacterial strains A4 and LBA9402 from Poland (A) and for the strain 8691 from Hungary (B), showing distinct bands at ca 1.0 kilobase. For the strains ATCC15834 of Poland and ATCC 15834 of Hungary, no clear amplification of the respective *rolB* gene could be accomplished. Amplification from the strain from Poland led to multiple bands that may be caused by unspecific binding of the primers. For ATCC15834 from Hungary, no amplified PCR product could be visualized by gel electrophoresis. The hypothesis that the absence might be related to probable inconsistencies due to high density of the initial bacterial suspension of ATCC15834 of both sources can be refuted. Although, growth determination of both ATCC15834 strains exhibited a relatively rapid increase in optical density, nevertheless, two dilutions of 1:10 and 1:100 were tested in multiple runs showing no PCR product.

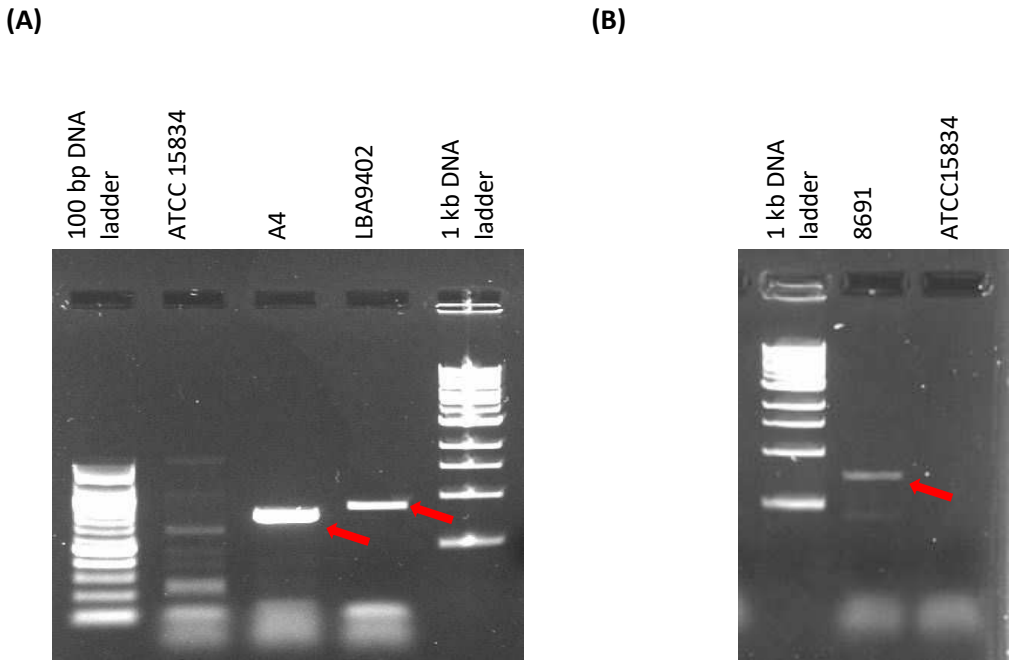


FIGURE 13 | Detection of *rolB* gene sequence. **(A)** *RolB* specific bands could be observed for bacterial strain A4 (arrow) and LBA9402 (arrow) from Poland below the 1.0 kilobases marker (1 kb DNA ladder). The sample ATCC15834 showed multiple bands that may be caused by DNA denaturation or unspecific primer binding. **(B)** A specific band was shown by bacterial strain 8691 (arrow) from Hungary, as well, whereas the presence of *rolB* gene for ATCC15834 could not be visualized.

5.3.2. Growth determination of *Rhizobium rhizogenes* strains

Growth of bacterial cultures was determined by means of spectrophotometry. The main goal was to obtain bacterial suspensions with an optical density at a wave length of 600 nm (OD_{600}) between 0.6 and 0.8. For this purpose, 20 mL of YEB medium was inoculated with various aliquots of bacterial overnight suspension and incubated overnight on a incubator shaker at 28°C and 200 rpm. The following day, every 2 hours samples were drawn and measured by spectrophotometry.

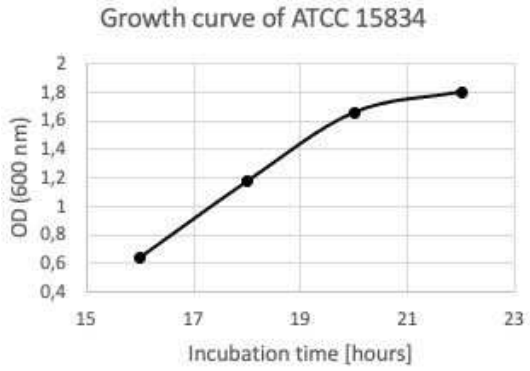
For *R. rhizogenes* strains ATCC15834, A4 and LBA9402 from Poland, varying growth rates of each strain were observed and led to different incubation times to accomplish the targeted optical density. Strain ATCC15834 reached the corresponding value with an inoculation aliquot of 1 µL after ca 17 hours, as depicted in figure 14 (A). Strains LBA9402 and A4 revealed a slower growth, so that after inoculation with an aliquot of 150 µL and 10 µL an incubation time of 26 hours and 27 hours, respectively, was necessary, as seen in figure 14 (B) and (C).

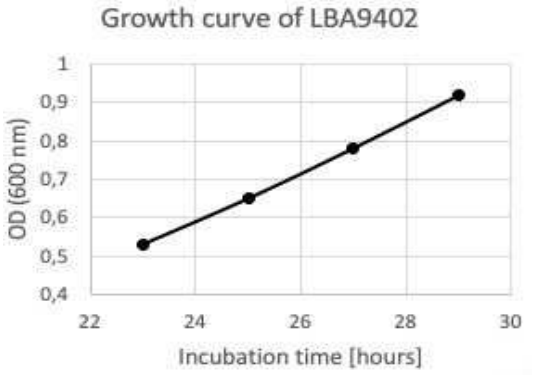
(A)

	Incubation time [hours]			
	16	18	20	22
OD_{600} of ATCC15834	0,645	1,178	1,660	1,804

(B)

	Incubation time [hours]			
	23	25	27	29
OD_{600} of LBA9402	0,532	0,651	0,782	0,92





(C)

	Incubation time [hours]			
	27	29	31	33
OD_{600} of A4	0,686	1,017	1,364	1,671

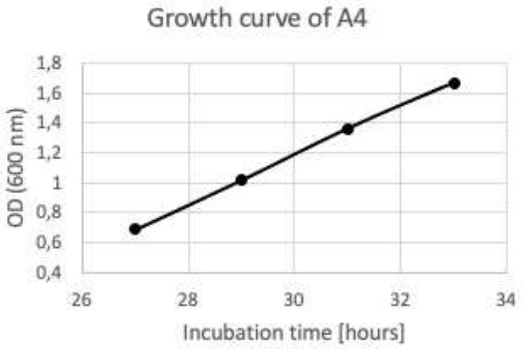


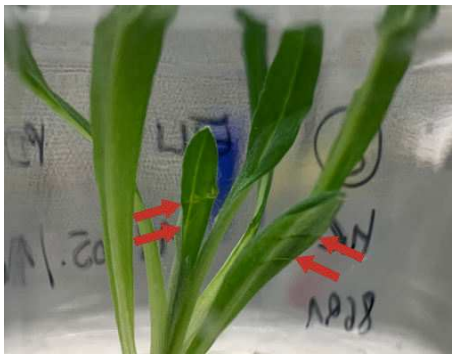
FIGURE 14 | Growth curves of *R. rhizogenes* strains from Poland. **(A)** Optimal incubation time for strain ATCC15834 was ca 17 hours. **(B)** Strain LBA9402 exhibited a slower growth resulting in an optimal incubation time of ca 26 hours. **(C)** Strain A4 showed an optimal incubation time of ca 27 hours.

For *R. rhizogenes* strains ATCC15834, A4 and 8691 from Hungary, previous experiments by Wöber showed that an OD_{600} of 0.6 to 0.8 was reached after approximately 17 hours of incubation at 28°C and 200 rpm by inoculating 20 mL LB medium with 1 µL of overnight bacterial suspension (Wöber, 2022).

5.3.3. Infection experiments of plant material

The aim was to infect the leaves of Edelweiss plantlets with bacterial strains of *R. rhizogenes* to induce hairy root formation. Infection experiments were conducted between May and August 2022 with a total amount of over 230 shoots of Edelweiss. For infection, bacterial overnight suspensions of the strains ATCC15834, A4 and 8691 from Hungary and ATCC15834, A4 and LBA9402 from Poland (see chapter 4.8.) were utilized. As the main strategy, 207 infections were carried out by inflicting cut wounds in the middle vein of leaves with a scalpel, as indicated in figure X (A), whereas 24 infections were carried out by puncturing leaves with a needle. Both the utilized scalpel and the needle were previously dipped in the respective bacterial suspension of *R. rhizogenes* that was supplemented with 50 µM of acetosyringone. For the experiments, an adopted protocol of Santos et al. (2005) was followed. The infected plantlets were transferred to the semi-solid 0.5 MS medium and kept under standard conditions. The cultures were checked frequently for hairy root formation which, according to Ondratschek (2012) and Hook (1993), should be visible 3-7 weeks after infection. After 8 weeks none of the infected plantlets showed hairy root formation. After further cultivation until week 12 still no signs of root formation were visible and the cultures were discarded. About 4 weeks after infection, wounded leaves started to show signs of withering, as shown in figure 15 (B). When wilted leaves came into contact with the culture medium, bacterial overgrowth could be observed, most likely due to the *Rhizobium* bacteria applied during the infection treatment. However, despite of a thick (ca 0.5 cm) bacterial layer, plant growth was not visually impaired.

(A)



(B)

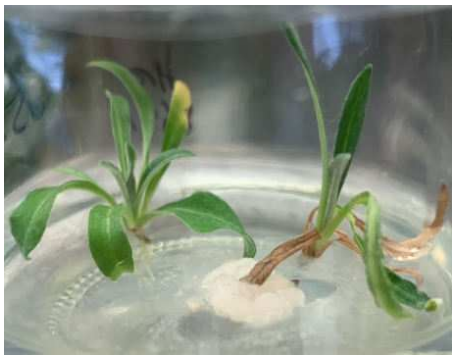


FIGURE 15 | Infection experiments by cutting wounds with scalpel dipped in bacterial suspension of *R. rhizogenes* strains ATCC15834, A4, 8691 and LBA9402. **(A)** The red arrows indicate the infection wounds immediately after incision. **(B)** Infected plantlets exhibited withering ca 4 weeks after infection that led to dried brown leaves. Bacterial contamination of the culture medium with *R. rhizogenes* was subsequently observed.

Concerning the second strategy, cut leaf explants of Edelweiss plantlets were used for infection, as described by Saleh and Thuc (2009) and Mahendran et al. (2022). In total, 14 cultures of Edelweiss explants with 8-10 excised leaves per vessel were prepared from June to August 2022. For infection, bacterial suspension of strains ATCC15834, A4 and LBA9402 from Poland and strain A4 from Hungary were utilized. The explants were submerged for 30 minutes in the respective bacterial suspension, which was supplemented with 50 µM acetosyringone, and placed on semi-solid 0.5 MS medium. For co-cultivation, the cultures were split into to two groups: half of the batch was incubated under a 16 hours daylight rhythm, the other half in the dark for 3 or 5 days, respectively. After co-cultivation the cultures were rinsed several times with an aqueous solution of cefotaxime (500 mg/L) and transferred to fresh semi-solid 0.5 MS medium supplemented with 500 mg/L cefotaxime.

About 1 week after infection, some leaf explants started to exhibit a pale or brown coloring, as depicted in figure 16 (A). After 2 weeks, withering of virtually all excised leaves could be observed, as shown in figure 16 (B), regardless if co-cultivation was carried out under light, or dark conditions. The experiments were terminated after 8 weeks.

(A) **(B)**

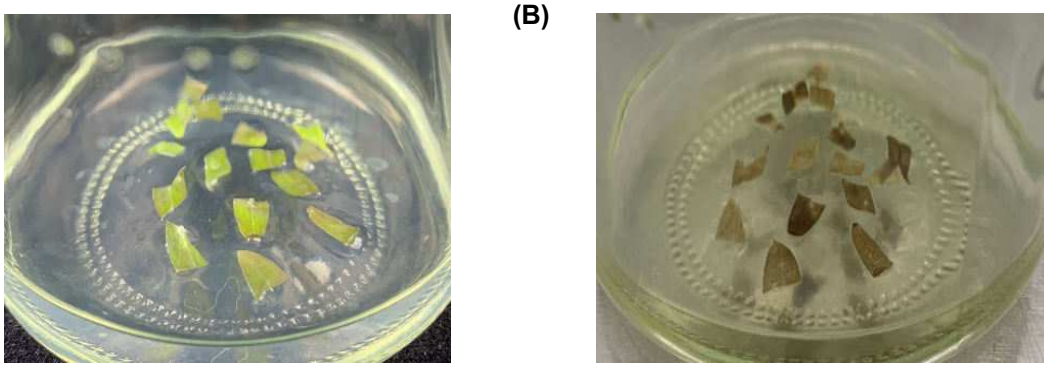


FIGURE 16 | Infection experiments with excised leaf explants that were incubated in the bacterial suspension of *R. rhizogenes* strains ATCC15834, A4, 8691 from Poland and A4 from Hungary. **(A)** Explants 8 days after infection showing fading and brown discoloration. **(B)** Withered explants 4 weeks after infection.

5.4. HPLC Analysis of dried hairy root samples of *Atropa belladonna* L.

Due to the fact that the infection experiments with Edelweiss turned out to be not successful and no hairy root formation was consequently observed, dried hairy root samples of *Atropa belladonna* L., obtained from the previous Master's Project of Wöber (2022), were used and analyzed regarding their content of atropine and scopolamine.

5.4.1. External standards and calibration curves

External standards of atropine and scopolamine hydrobromide trihydrate were utilized for identification and quantification of the alkaloids in the hairy root samples. To validate the suitability of the HPLC method, calibration curves of atropine and scopolamine hydrobromide trihydrate were generated. For acquiring data, 6 methanolic dilutions per external standard were prepared and subjected to HPLC analysis. The mean peak areas were then plotted against the corresponding concentrations and a trend line was generated to assess the coefficient of determination (R^2). Both calibration curves are shown in figure 17 (A) and (B). The coefficient of determination of 0.9949 for atropine and 0.9979 for scopolamine hydrobromide trihydrate was calculated. In consequence, the linear correlation of peak area and content could be confirmed which in turn ensured the suitability of the utilized quantification method.

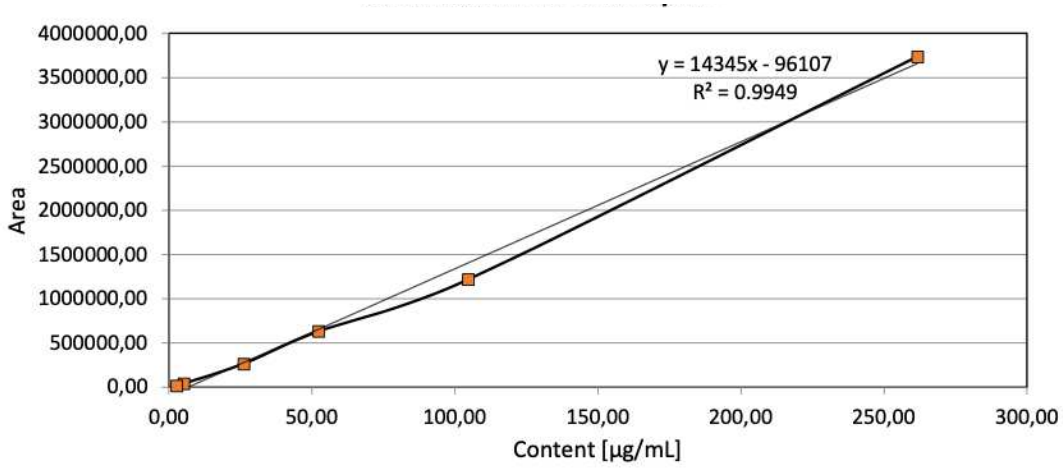


FIGURE 17 (A) | Calibration curve of atropine. Mean peak areas were plotted against the concentration of 261.67, 104.67, 52.33, 26.17, 5.23 and 2.62 µg/mL.

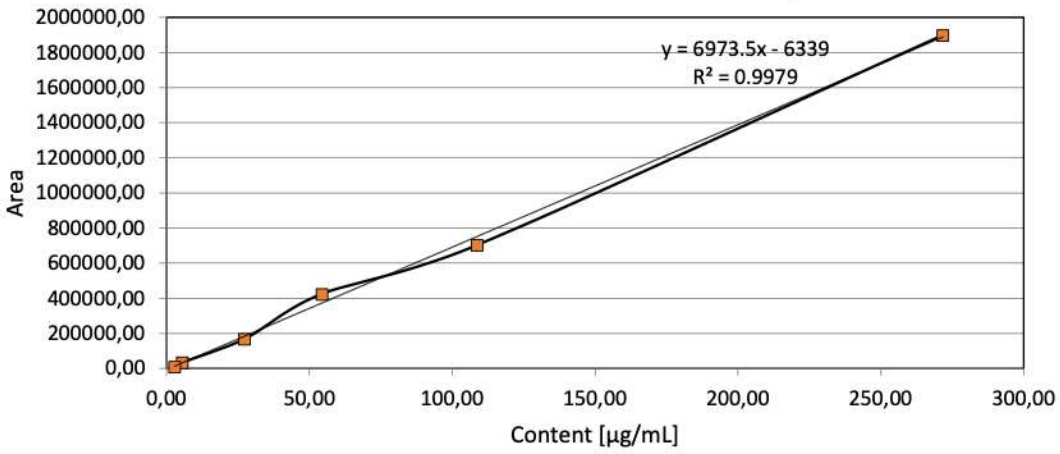


FIGURE 17 (B) | Calibration curve of scopolamine hydrobromide trihydrate. Mean peak areas were plotted against the concentration of 271.67, 108.67, 54.33, 27.17, 5.43, 2.72 µg/mL.

5.4.2. Alkaloid contents in hairy root samples

The content of atropine and scopolamine in hairy roots extracts was determined by HPLC, using external standards of atropine and scopolamine hydrobromide trihydrate. Processing of the hairy roots and preparation of standard solutions are described in chapter 4.9.3. The HPLC injection volume was 10 µL and all measurements were carried out in triplicate. The resulting peaks of atropine and scopolamine were identified visually by comparing the retention time of the respective external standard with the HPLC chromatogram of the hairy root samples. The retention times were ca 10.59 minutes for atropine and ca 9.57 minutes for scopolamine. Calculation of the alkaloid contents in the samples was performed with MS Excel. The resulting data are presented in table 3.

TABLE 3 | Content and standard deviation of atropine and scopolamine per hairy root sample.

Sample number	Sample name	Content of atropine		Content of scopolamine	
		µg/g	SD	µg/g	SD
1	AB-A4-34	577,56	51,45	279,10	24,43
2	AB-A4-66	227,00	3,22	123,32	2,47
3	AB-A4-36	481,50	39,45	117,75	9,12
4	AB-A4-41	606,09	15,85	133,00	3,91
5	AB-A4-69	768,45	9,60	89,44	2,04
6	AB-A4-55	134,69	5,96	25,12	0,94
7	AB-A4-47	339,36	6,12	196,62	4,53
8	AB-A4-48	252,34	5,51	228,92	5,93
9	AB-A4-33	300,14	10,56	82,06	1,18
10	AB-A4-62	496,26	9,95	239,92	3,19

As illustrated in figure 18, both atropine and scopolamine were detected in all 10 hairy root samples. They exhibited an average content of 418.34 µg/g of atropine and 151.53 µg/g of scopolamine.

In hairy root line AB-A4-69 the highest content of atropine was detected with 768.45 µg/g, followed by AB-A4-41 and AB-A4-34 with 606.09 µg/g and 577.56 µg/g, respectively. The lowest atropine content was found in line AB-A4-55 with 134.69 µg/g, which was 5.7 fold lower than in line AB-A4-69. Concerning the content of scopolamine, hairy root line AB-A4-34 showed the highest value with 279.10 µg/g. Lines AB-A4-62 and AB-A4-48 contained slightly lower amounts of 239.92 µg/g and 228.92 µg/g, respectively. Line AB-A4-55 exhibited the lowest content in scopolamine (25.12 µg/g), which was 11.1 fold lower than in line AB-A4-34. The highest total alkaloid content (= sum of atropine and scopolamine) was found

to be 857.89 µg/g in hairy root line AB-A4-69, with an atropine to scopolamine ratio of 8.6. The lowest alkaloid content was detected in line AB-A4-55 with 159.82 µg/g and a ratio of 5.4. In all 10 analyzed hairy root samples, the content of scopolamine never surpassed the value of atropine. The highest atropine to scopolamine ratio (8.6) was measured in line AB-A4-69, which was more than twice as high as the average ratio of all 10 samples (3.5). In comparison, the lowest atropine to scopolamine ratio (1.1) was found in line AB-A4-48.

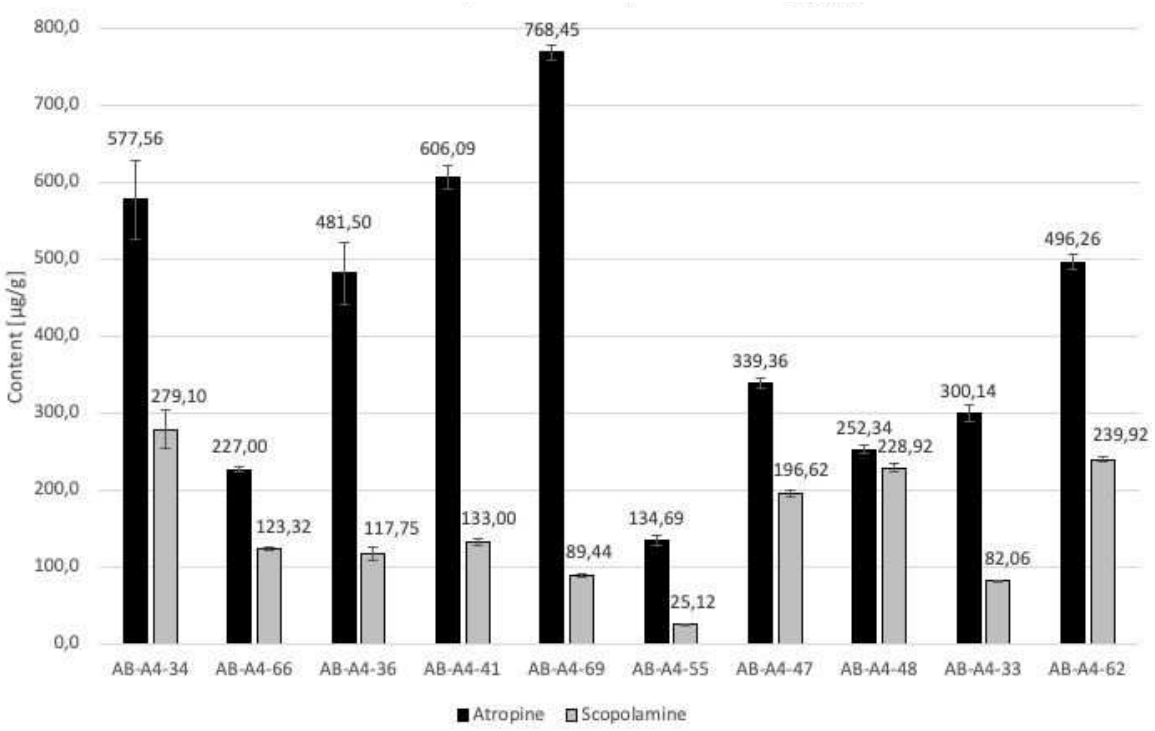


FIGURE 18 | Content of atropine and scopolamine per analyzed hairy root line.

6. Discussion

In vitro production of pharmaceutically bioactive secondary metabolites, especially utilizing hairy root culture techniques, could ensure a consistent and controllable production, reliable reproducibility and easy upscaling options (Wawrosch and Zotchev, 2021). However, it also shows considerable difficulties and limitations, including low efficiency of the production system, large technical effort, and legal restraints. Due to the low content of secondary metabolites often found in medicinal plants, even semisynthetic modifications of such compounds can be associated with unsustainably high costs (Wawrosch and Zotchev, 2021). To date, the only industrial process involving production of secondary metabolites via plant cell culture technology is the production of the antitumor agent Taxol® (INN: paclitaxel). For two decades now, cell suspension cultures of *Taxus chinensis* are used for the manufacture of paclitaxel on a large scale (Imseng et al., 2014). While plant *in vitro* technologies most likely have the potential for the future, further efforts need to be devoted to maximize productivity of such biotechnological approaches.

Previous studies described the application of *in vitro* hairy root culture technique on *Leontopodium nivale* subsp. *alpinum* (Hook, 1993; Wawrosch et al., 2014; Ondratschek, 2012). For this work, it was initially planned to investigate changes in the content of selected secondary metabolites of Edelweiss hairy root cultures upon elicitation with Edelweiss-specific bacterial endophytes. The establishment of *in vitro* shoot cultures of Edelweiss was initially attempted by aseptic germination of Edelweiss seeds and further cultivation including propagation via side shoots (see chapter 4.4.). Due to the high, obviously seed-originated, microbial contamination of most samples of the initiated seed cultures, a second strategy was employed to achieve the necessary amount of plant material. This was accomplished by further propagation of Edelweiss shoot cultures deriving from clonal lines that were initiated in 2020.

In this work, prior to cultivation, seeds were surface sterilized using an aqueous solution of sodium hypochlorite according to the sterilization protocol of Wawrosch et al. (2014). The sterilization treatments revealed an unusually high occurrence of seed-originated microbial contamination leaving 91.3% of the seed cultures contaminated, whereas only 8.7% were sterile. As treatment conditions, a 25-50% (v/v) aqueous solution of sodium hypochlorite and an exposure time of 30-50 minutes were employed. The highest contamination rate (100%) was observed with the seeds of Jelitto GmbH. Previously, Ondratschek (2012) reported lower contamination rates up to a maximum of 50% using a 20-25% (v/v) aqueous

sterilization solution and an exposure time of 25-30 minutes, most contaminations also occurred with the seed material of Jelitto GmbH. In the studies of Hook (1993) a 10% aqueous solution of sodium hypochlorite was employed for 20 minutes and was observed to be effective. Regarding the sterilization parameters, 0.5-5% (v/v) sodium hypochlorite or 3-7% (v/v) of an aqueous solutions of calcium hypochlorite for 10-30 minutes, respectively, are in general recommended for surface sterilization of plant parts or whole plants (Illiev et al., 2010).

The results of our experiments suggest that the outcome of the surface sterilization treatments may strongly depend on the amount of seed-borne microorganisms (i.e. not just the microorganisms adhering on the seed surface). In most cases a substantially lower concentration of sodium hypochlorite, like e.g. 10% or less, should be sufficient to surface sterilize most of the seed material. This was also verified by incubating samples of washing water and rubber bands that were used for surface sterilization, as depicted in chapter 5.1. No microbial contamination in any experiment conducted was observed. The application of alternative methods such as using chlorine gas for disinfection, as described by Altpeter and Sandhu (2010), might be an interesting approach that needs to be investigated for Edelweiss seeds.

The bacterial strains ATCC15834, A4, LBA9402 and 8691 of the soil-inhabiting Gram-negative bacterium *Rhizobium rhizogenes*, formerly known as *Agrobacterium rhizogenes*, were used in over 230 infection experiments with Edelweiss plantlets. Prior to infection, the strains were examined regarding their capability of plant cell transformation by verifying the presence of the *Rhizobium* specific *rolB* gene (see chapter 5.3.1.), and growth curves were compiled to determine the parameters for optimal suspension preparation. None of the experiments led to hairy root formation within 12 weeks after infection. Previous studies on Edelweiss showed that hairy root formation occurred within 4-6 weeks with the bacterial strains ATCC15834, LBA 9402 and TR105 (Ondratschek, 2012), and after 7 weeks with a kanamycin-resistant strain of *R. rhizogenes* that was not further described (Hook, 1993). Due to the comparable conditions under which the infection experiments were executed, possible reasons for the failure of our experiments cannot be clearly identified.

Previous research indicates that transformation frequency strongly depends, among other factors, especially on the virulence of the *Rhizobium* strain utilized. Well-documented data reported that the strains ATCC15834, LBA 9402 and A4 show higher transformation frequency than others (Gantait and Mukherjee, 2021). On the other hand, especially plant species from the families of Araliaceae, Asteraceae and Solanaceae show stronger inducibility

of hairy root formation (Gantait and Mukherjee, 2021). Over the past decades, hairy root formation following the infection with *R. rhizogenes* bacteria was described for more than 100 plants, revealing that some strains show high virulence towards certain plant species. ATCC15834 is one of the more frequently used *Rhizobium* strains that was first characterized in the 1980s showing high virulence. Its genome contains three plasmids (pAr15834a, -b and -c) of which pAr15834b was determined to be the root-inducing plasmid (pRI) that is essential for successful plant transformation (Kiryushkin et al., 2021).

A common method to increase bacterial virulence in *R. rhizogenes* is the addition of acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone) to the bacterial suspension before infection. Acetosyringone is an endogenous secondary plant metabolite that is released when plant tissue is wounded. It is presumably recognized by phenol-binding proteins of *Rhizobium* that stimulate the expression of specific *vir* (virulence) genes (Baker et al., 2005). In this work 50 µM of acetosyringone were added to the bacterial suspension immediately before use. Previous studies determined a concentration between 50 and 200 µM as most effective to enhance transformation frequency tested on different plant species (Mahendran et al., 2022; Bhagat et al., 2019; Saleh and Thuc, 2009; Grzegorczyk-Karolak et al., 2006; Kumar et al., 2006). The study of Grzegorczyk-Karolak et al. (2006) on hairy root cultures of *Salvia officinalis* L. revealed that with *Rhizobium* strain A4 the induction frequency could be increased by 30% when 200 µM of acetosyringone were added to the bacterial medium before incubation. The addition of acetosyringone to strain ATCC15834, in turn, resulted in a decrease of induction frequency. However, in her study on Edelweiss Ondratschek (2012) achieved hairy root formation even without the usage of acetosyringone, which in turn indicates that in our experiments the infection should have been successful under the conditions mentioned above.

Aside from virulence, other factors that might have an impact on transformation frequency would include preparation of bacterial suspension, co-cultivation conditions, stage of plant development, specific type of explant and more. Concerning the preparation of bacterial suspensions, in this work 20 mL of liquid medium (see chapter 4.8.) were inoculated with an aliquot of overnight bacterial suspension and incubated on a incubator shaker until the suspension reached an OD_{600} of 0.6-0.8. Similar optical densities turned out to be efficient for other plant species, such as for example, for *Solenostemon scutellarioides* (Saleh and Thuc, 2009), *Raphanus sativus* L. (Muthusamy and Girija, 2020) and *Swertia chirayita* (Mahendran et al., 2022).

Future studies should first of all focus on the improvement of the sterilization protocol. For example, the inclusion of a treatment with 70% ethanol for 1 minute prior to surface sterilization was reported (Neumann et al., 2020). An alternative interesting approach for sterilization of plant material should also be included at this point that was employed for seeds of bahiagrass. For surface sterilization, 20 mL of a 6% (v/v) sodium hypochlorite solution was carefully mixed with 10 mL glacial acetic acid in a beaker glass and placed in the bottom compartment of a desiccator. The seeds were transferred to the upper level of the desiccator in an open petri dish and incubated for 1 hour in the closed system (Altpeter and Sandhu, 2010), so that evaporated chlorine gas from the bottom containment disinfected the seed surface.

Furthermore, *Rhizobium* suspensions with higher densities, combined with concentrations of acetosyringone above 50 µM, should be tested for hairy roots induction in Edelweiss. Due to the fact that the use of excised leaf explants of Edelweiss turned out to be not successful in our work, and also in the study of Ondratschek (2012), alternative protocols should be considered and comprehensively assessed. Kumar et al. (2006) presented an interesting approach using ultrasonication for infection of plant material. Their results show that transformation frequency was increased by 25% with sonication and was further increased by 65% by supplementing the *Rhizobium* culture with 100 µM of acetosyringone. Another alternative protocol described the beneficial effect of pre-culturing the excised leaf explants for 2 days prior to infection. The procedure led to less issues concerning withering of the explants (Bhagat et al., 2019). Leaf explants of Edelweiss would presumably benefit from this procedure as well. During the infection experiments, it was frequently observed that a few days after infection the explants gradually started to wither. This interim step should also be considered for further experiments with Edelweiss due to the fact that excised explants tend to dehydrate quickly.

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