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„Endophyte isolation from Edelweiss (*Leontopodium nivale* subsp. *alpinum*)“

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Abbreviation

2xYT .....twofold Yeast Extract / Tryptone
BLAST .....Basic Local Alignment Search Tool
Cass. .....Alexandre Henri Gabriel de Cassini
DMSO .....Dimethyl sulfoxide
dH_2O.....Deionized water
DNA.....Deoxyribonucleic acid
dNTPs .....Deoxy nucleotide triphosphates
EtOH .....Ethanol
FW.....Forward
gDNA.....Genomic deoxyribonucleic acid
GPS.....Global position system
$HgCl_2$ .....Mercuric (II) chloride
HV .....Humic acid vitamin
IUCN .....International Union for Conservation of Nature
kb .....Kilo base
kbp .....Kilo base pairs
MEGA.....Molecular Evolutionary Genetics Analysis
NaOCl .....Sodium hypochlorite
NC .....Combination of the antibiotic's nystatin and cycloheximide
nt .....Nucleotides
PCR.....Polymerase chain reaction
PDA.....Potato dextrose agar
Pfu polymerase .....Pyrococcus furiosus polymerase
rRNA .....Ribosomal ribonucleic acid
RV .....Reverse
Taq polymerase.....*Thermus aquaticus* polymerase
TSA .....Tryptic soy agar
TSB .....Tryptic soy broth
TBE .....Tris / Borate / EDTA

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Abstract

Endophytic microorganisms are rich in biodiversity and reside inside the living plants without causing any harm to the host. In every vascular plant species studied to date at least one endophyte could be found. Since the discovery of a Taxol-producing endophyte, the worldwide scientific effort on isolating endophytic microorganisms as novel source of natural products for medicinal, agricultural and industrial applications is ever growing.

In the current work, five individuals of the traditional medicinal plant *Leontopodium nivale* subsp. *alpinum* were collected form the native alpine region Rax. A surface sterilization protocol for plant *in vitro* cultivation as well as for endophyte isolation was established. Furthermore, a maceration protocol was developed for three different tissue types of Edelweiss, specifically leaves, rhizomes and roots. Six selective media, three of them supplemented with antibiotics for additional selective pressure, were used for the isolation of microorganisms. Isolated endophytic bacteria were cultivated, and the genomic DNA from some endophytes were extracted using the boiling method. 16S-rDNA gene fragments were amplified from these DNAs, sequenced by Sanger sequencing method and subjected to phylogenetic analysis.

The second focus in this study was the establishment of a tissue culture of rosettes and the callus induction of leaves from *Leontopodium nivale* subsp. *alpinum*. In future projects, endophytic microorganisms as elicitors will be tested for the enhancement of the production of bioactive compounds in plant cell suspension cultures.

Zusammenfassung

Endophytische Mikroorganismen sind ubiquitär verbreitet und haben eine reiche biologischen Vielfalt, sie besiedeln lebende Pflanzen ohne diese zu schädigen. In jeder vaskulären Pflanze, die bis heute untersucht wurde, wurde zumindest ein Endophyt nachgewiesen. Seit der Entdeckung eines Taxol-produzierenden Endophyten in der pazifischen Eibe ist das Bestreben der Wissenschaft, endophytische Organismen zu isolieren, ungebrochen. Endophyten haben großes Potential als neuartige Wirkstofflieferanten für medizinische, landwirtschaftliche und industrielle Zwecke.

Fünf Individuen von *Leontopdium nivale* subsp. *alpinum* wurden auf dem Raxplateau, dem natürlichen Vorkommen dieser traditionell medizinisch genutzten Pflanze gesammelt. Im Focus dieses Projektes stand die Etablierung eines Sterilisationsprotokolls, das sowohl für die pflanzliche Gewebekultur als auch für die Isolation von Endophyten verwendet werden kann. Weiters wurde ein Mazerationsprotokoll für drei unterschiedliche Pflanzengeweben von Edelweiß, im speziellen für Blätter, Rhizome und Wurzeln, angefertigt. Sechs verschiedene Selektivnährböden, drei wurden zusätzlich mit Antibiotika versetzt, wurden für die Isolation endophytischer Mikroorganismen verwendet. Die isolierten Endophyten wurden kultiviert und es wurde begonnen die genomische DNA mittels „Aufkoch“-Methode zu extrahieren. Die 16S-rDNA wurde vervielfältigt und mit der Didesoxymethode nach Sanger sequenziert.

Neben der Isolierung der Endophyten stand die Etablierung der pflanzlichen Gewebekultur der Rossetten, als auch die Etablierung einer Kalluskultur der Blätter von *Leontopodium nivale* subsp. *alpinum* im Fokus.

In zukünftigen Projekten sollen die endophytischen Mikroorganismen als Elizitoren zur Erhöhung der Sekundärmetabolite in pflanzlichen Suspensionskulturen getestet werden.

Introduction

Leontopodium nivale subsp. *alpinum*

The herbaceous plant Edelweiss, *Leontopodium nivale* subsp. *alpinum* (Cass.) Greuter, syn. *Leontopodium alpinum*, belongs to the family Asteraceae and occurs in subalpine to alpine vegetation in the central European mountain ranges and occurs predominantly on limestone dry steppes and mostly on limestone cliffs (Dobner, Schwaiger, et al. 2003; Keller and Vittoz 2015). The center of diversity of the genus *Leontopodium* is the Sino-Himalayan region in south-western China, which harbors 30–41 species (Blöch et al. 2010; Safer et al. 2011). After the last ice age, *Leontopodium nivale* subsp. *alpinum* has colonized to mountainous regions of Europe and is now distributed from the mountains of Balkan in the east and the Pyrenees in the west, specifically the Balkan Mountains, the Tatra, the Alps, the Carpathians and the Pyrenees (Erhardt 1993; Pace, Bruno, and Spanò 2009; Safer et al. 2011).

Leontopodium nivale (Ten.) Huet ex Hand.-Mazz is the only indigenous species of the genus *Leontopodium* in Europe and *Leontopodium nivale* subsp. *alpinum* an infraspecific taxa of it (Greuter 2006, The Plant List 2013).

Many studies aimed to clarify the taxonomic relationship between *L. alpinum* and *L. nivale*. However, it is still unclear whether they are two different species (Blöch et al. 2010) or a single species with two subspecies (Greuter 2003; Safer et al. 2011). The different taxonomic approaches caused a confusing nomenclature in publicly available literature. An amplified fragment length polymorphism (AFLP) study with a large number of European genotypes would be necessary to solve

Figure 1: Flowers of *Leontopodium nivale* subsp. *alpinum*

this problem (Safer et al. 2011). According to the online database “The Plant List” *Leontopodium nivale* subsp. *alpinum* is the accepted name and *Leontopodium alpinum* is its synonym (The Plant List 2013).

From now on in this study only the name *Leontopodium nivale* subsp. *alpinum* will be used.

Edelweiss is a hemicryptophyte and blooms between July and September (Ischer et al. 2014). Due to the fact that Edelweiss grows on high altitudes and is exposed to strong UV radiation, the whole plant is covered with white trichomes (Kertész et al. 2006). This wooly layer limits water evaporation from the plant, so Edelweiss can tolerate long drought periods (Vigneron et al. 2005). The leaves are lanceolate, densely felted and create a rosette on the ground with each one flowering stalk. *Leontopodium nivale* subsp. *alpinum* is up to 20 cm high perennial herb and has a star-like inflorescence consisting of 4–12 yellowish capitula surrounded by white and woolly bracts. After blooming capitula form 1 mm long, oblong achenes equipped with a pappus (Erhardt 1993; Keller and Vittoz 2015; Pace et al. 2009; Vigneron et al. 2005).

The genus name *Leontopodium* (lion´s paw) comes from Greek leon (for lion) and podion (for foot) (Hegi 1965). Edelweiss is German for nobel (edel) and white (weiss), inspired by the ornamental flower (Dweck 2004; Milic 2012).

Leontopodium nivale subsp. *alpinum* is a symbol of the Alps which has vanished from many localities suffering from being a tourist and botanist attraction (Erhardt 1993; Muica; Popoca-Cucu 2016). Nowadays, the species is protected in many countries and regions (Keller and Vittoz 2015).

Plants always have been an important source of therapeutic agents and play a major role in discovery of drugs (Al-Rubaye, Hameed, and Kadhim 2017; Cragg, Newman, and Snader 1997; Koehn and Carter 2005). Since ancient times, Edelweiss was a part of the cultural heritage of the European Alps. Furthermore, the use in traditional folk medicine is common since a long time (Dobner et al. 2004; Safer et al. 2011; Tauchen and Kokoska 2017). Edelweiss is mentioned in historical references for the treatment of different diseases like angina pectoris, fever, bronchitis, diarrhea, and cancer in humans as well as in livestock (Dobner et al. 2004).

For a long time, there was hardly anything known about the phytochemistry of Edelweiss (Chiej 1984). Through the enormous popularity and the evidences of traditional use, Edelweiss became a focus of scientific attention (Tauchen and Kokoska 2017).

For several years, groups around the world have investigated Edelweiss' secondary metabolites and their biological activity. Identified compounds of Edelweiss are flavonoids, phenolic acids, tannins, coumarins, sesquiterpenes, benzofuran and benzopyrane (Comey et al. 1999; Dweck 2004; Ganzera et al. 2012; Schwaiger et al. 2006).
In 2003, Dobner et al. examined *Leontopodium nivale* subsp. *alpinum* for its antibacterial activity, due to its broad range of applications in folk medicine. The growth inhibiting effect extends over Gram-positive as well as Gram-negative bacteria. The effect of dichloromethane extracts of the aerial parts and the roots covers a significant inhibition of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes* strains. Linolic and linoleic acids have been identified as the most effective secondary metabolites regarding antimicrobial activity, both inhibiting a multi-resistant strain of *Staphylococcus aureus*.

The antioxidative efficacy of leontopodic acid, a major compound in the aerial parts of Edelweiss, has been demonstrated with various *in vitro* methods. Leontopodic acid has a protective effect on DNA, it counteracts damage by free radicals and the antioxidant potential is much more pronounced, than those of silymarin and chlorogenic acid (Schwaiger et al. 2005; Tauchen and Kokoska 2017). Silymarin, from milk thistle, and chlorogenic acid are known as highly potent antioxidants and their efficacy is reported in many studies (Huang et al. 2007; Shaker, Mahmoud, and Mnaa 2010). Furthermore, leontopodic acid improves and strengthens the skin barrier, reduces skin sensitivity and protects skin surface from environmental factors. Hence, extracts of *Leontopodium nivale* subsp. *alpinum* are commonly used in anti-aging products and other cosmetics (Mistry 2017). Kostyuk et al. (2018) demonstrated that leontopodic acid has the potential to reduce the UV-A and UV-B related skin damage, which makes it attractive for use in sun screening creams. Due to the increasing demand of Edelweiss extracts for the cosmetic and food industry, a breeding program for

Edelweiss was started. The hybrid Edelweiss ‘Helvetia‘ has a high level of leontopodic acid and a high dry weight (Vouillamoz et al. 2009).

Leoligin, the major lignan of the roots of Edelweiss, is a very anti-inflammatory and anti-proliferative secondary plant metabolite and was first isolated at the University of Innsbruck (Reisinger et al. 2009). According to Reisinger et al. (2009), leoligin inhibits intimal hyperplasia after venous bypass grafts and leukotriene biosynthesis. Vascular diseases are precursors of arteriosclerosis and therefore major causes of cardiovascular disease. Leoligin reduces existing hyperplasia of blood vessels, stent implants coated with leoligin prevent restenosis without toxic side effect.

Roots of Edelweiss contain only a small amount of leoligin, thus for isolation of 40 mg leoligin, 800 g of air-dried roots are needed. Wawrosch et al. (2014) successfully established a transformed hairy roots culture that was able to produce an increased amount of leoligin.

Endophytes

Several hundred million years ago – with the first appearance of higher plants – the relationship between plants and microorganisms may have developed. Investigation of fossilized stems and leaves have indicated the existence of plant-associated microbes (Gary A.Strobel 2003; Zhao et al. 2010). Mutualistic symbiosis was also found in roots of *Amyelon radicans* from the Paleozoic era (ca 540-250 million years BC) (Hyde and Soytong 2008). Endophytes are microorganisms colonizing the intercellular and intracellular space of any plant tissue for the whole lifetime or just for a period of time, without causing harm to their host (Alvin, Miller, and Neilan 2014; Jalgaonwala, Mohite, and Mahajan 2011; Mei and Flinn 2009; Stone, Polishook, and White n.d.; Strobel 2003). The knowledge about the existence of endophytes can be traced back to first scientific evidences by Pasteur and others in 1870 (Hallmann et al. 1997). On the earth nearly 300.000 vascular plant species exist, from which each host at least one endophytic microbe (Ryan et al. 2008; Strobel et al. 2004; Tan and Zou 2010). Currently, scientists pay attention to plants as a reservoir of an outstanding number of microorganisms (Strobel G 2003; Tan and Zou 2010). Only a few

terrestrial and aquatic plants have been completely examined for their endophytic diversity as well as for the production of bioactive compounds (Khan et al. 2007; Ryan et al. 2008; Tan and Zou 2010). The probability to find novel and valuable endophytic microbes from different ecosystems and unique biological niches is considerable (M.P. Gutierrez, M.N. Gonzalez, and M. Ramirez 2012; Ryan et al. 2008). Plants can be associated with microorganisms in various ways, symbiotic lifestyles range from mutualism, commensalism through to parasitism (Kogel, Franken, and Hückelhoven 2006; Redman, Dunigan, and Rodriguez 2001). According to Álvarez-Loayza et al. 2011, it depends on the circumstances if an endophyte may be pathogenic or not.

Endophytes can be hosted in the below- and aboveground tissues of plants, called rhizosphere and endosphere (Frank, Saldierna Guzmán, and Shay 2017). There are three ways plants can acquire endophytes – vertically (direct transfer from generation to generation), horizontally (acquisition from the environment) or by combination of both mechanisms (Bright and Bulgheresi 2010; Frank et al. 2017).

Transmission routes of endophytes

Vertical transmission routes

Plant seeds host a limited range of vertically transmitted endophytic organisms. Plants formed mutualism with endophytes that are beneficial against pathogenicity (Mitter et al. 2017; Truyens et al. 2015). Endophytes can colonize the coat, the endosperm as well as the embryo of the seeds (Frank et al. 2017). Endophytic organisms enter the plant seeds in different ways. The transfer of an endophyte can occur from vegetative plant tissues to the seed via vascular connection either through the funiculus to the endosperm or through the micropyle, a small opening in the ovule through which spermatozoa can pass. Another way is the direct transfer of endophytes from gametes to endosperm and embryo. The vertical transmitted endophytes can colonize the shoot meristem, which are undifferentiated cells and become reproductive meristem, and can lately colonize the developing seeds. Finally, the transfer from fruits or pollen to

seeds is also possible (Frank et al. 2017; Puente, Li, and Bashan 2009; Truyens et al. 2015).

Horizontal transmission

Most endophytes derive from the soil and colonize the spermosphere and rhizosphere via horizontal transmission (Frank et al. 2017; Hallmann et al. 1997).

The spermosphere is the zone surrounding a germinating seed, where microorganisms interact with the germinating seed. When seeds start germinating, they release carbohydrates in the form of sugars. This is an attractive energy source for microorganisms, which start to colonize the spermosphere and enter the seedling within a few hours (Frank et al. 2017; Johnston-Monje and Raizada 2011; Nelson 2004; Schiltz et al. 2015).

The soil-root interface attracts microorganisms due to secretion of rhizodeposits and root exudates and increase the microbial population near roots. Plant root secreted compounds are involved in the colonization of the rhizosphere by microorganisms. Root exudates, photosynthates, amino acids and proteins increase the soil biota near the roots and steer the colonization process between host plants and endophytes (Frank et al. 2017; Hardoim et al. 2015; Kandel, Joubert, and Doty 2017). Moreover, endophytes themselves take an active role in colonizing the host plant. Motility, chemotaxis and quorum sensing are essential tools for the aggregation near the root surface or to outcompete neighboring microorganisms. The first step of colonization is the adhesion of microbial cells to the rhizoplane. Potential endophytic microorganisms can pass the internal plant tissue through openings in the roots and can finally colonize all plant compartments by distribution through the vascular system (Frank et al. 2017; Kandel et al. 2017).

Endophytes can also inoculate the aerial tissues of the host, termed the phyllosphere. This habitat includes fruits, flowers, leaves and stems. Most phyllosphere endophytes derive from bioaerosols, atmospheric particles emitted from the biosphere. Bioaerosols can contain living and dead organisms, virus particles, dispersal units like spores and pollen or plant debris (Frank et al. 2017; Fröhlich-Nowoisky et al. 2016). Atmospheric dispersion of organisms plays key role in genetic exchanges between habitats over long distances (Fröhlich-

Nowoisky et al. 2016; Yamaguchi et al. 2012). Dust from Sahara Desert in Africa can transport microorganisms to high altitude areas in Europe (Frank et al. 2017; Meola, Lazzaro, and Zeyer 2015). According to Joung, Ge, and Buie (2017) a single raindrop splashing on the soil surface can aerolize 0.01% of the bacteria. Little is known about the penetration of endophytes via openings in the epidermis of leaves, but studies show that pathogens can use stomata as a gateway into the plant aboveground parts (Frank et al. 2017). The stomata pore is surrounded by a pair of guard cells, the highly specialized cells that control relative humidity, gaseous exchange and the uptake of carbon dioxide (Cominelli et al. 2005; Frank et al. 2017). Moreover, stomata play a key role in plant survival as defense mechanisms against pathogens. The guard cells can prevent the penetration of pathogens by sensing microbe-associated molecular patterns (MAMPs), like flagellin or lipopolysaccharides, and close the pore of the stomata in response (Frank et al. 2017; Montillet and Hirt 2013). The fact that plants can recognize pathogens and prevent them from entering through stomata pores, raises the question whether beneficial organisms are allowed or recruited to enter the aerial parts as endophytes. There are some reports of endophytes colonizing the plant via stomata, for example *Herbaspirillum seropedicae* in *Ananas comosus* (pineapple) or *Bacillus amyloliquefaciens* in *Vanilla phaeantha* (vanilla orchid) (Baldotto, Olivares, and Bressan-Smith 2011; Frank et al. 2017; White et al. 2014).

Wind-transported pollen can also be involved in the horizontal transmission of endophytes. Pollen grains can be transported over long distances by the wind or pollinators, so the environmental exposure is given. Microorganisms can be acquired from the atmosphere or via animals.

Beside pollen of wind-pollinated species, pollinators and other flower visiting insects are highly relevant transmission routes for microbial communities in floral tissue (Frank et al. 2017). Plant-feeding insects (i.e. cicardellidae, psyllids, sap-feeding insects) can transmit numerous pathogens by puncturing the phloem or xylem vessels. A recent study demonstrated the transmission of the endophytic microbial community from one grapevine plant to others by insect vectors (Frank et al. 2017; Lòpez-Fernàndez et al. 2017, Harris and Maramorosch 1980).

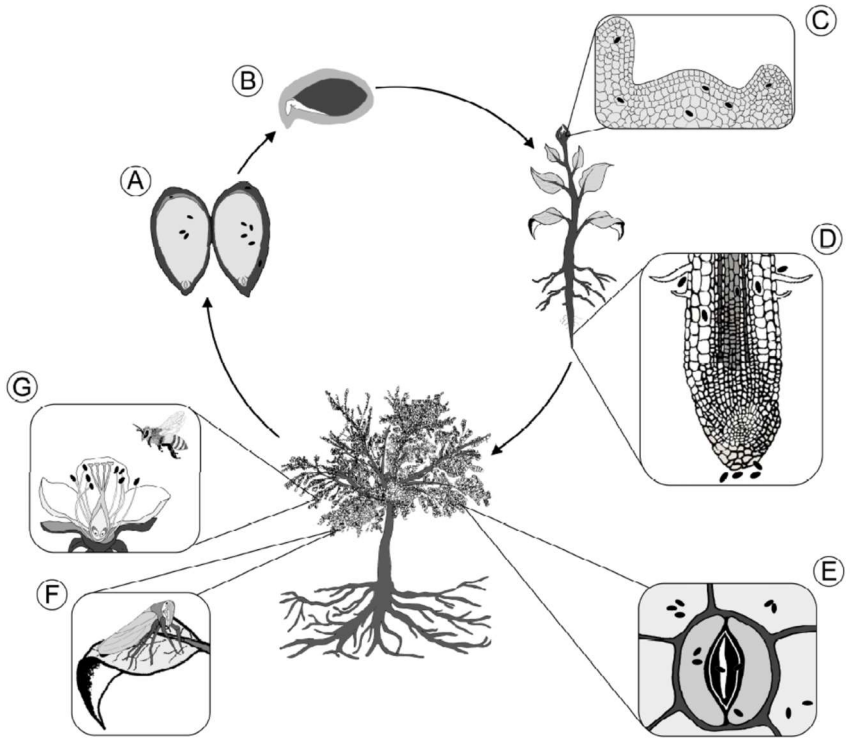

Figure 2: Transmission routes of endophytic microorganisms
[A] Vertically via seeds [B] Colonization of the spermosphere (grey area around the seed) [C] Developing reproductive organs were colonized via apical meristem [D] Transmission from soil to roots [E] Colonization through stomata of the leaves via the air [F] Colonization via sap-feeders [G] Colonization via pollinators (Frank et al. 2017).

Physiological and ecological roles of endophyte-host interaction

The interaction between endophytes and host plant provides beneficial effects for both (Mei and Flinn 2009). Plants in symbiosis with endophytic microbial communities are often healthier than plants without endophytes (Zhang, Song, and Tan 2006).

Endophytes can produce phytohormones, modulate the phytohormone levels of the plants or produce other growth-promoting substances (Schmelz et al. 2016; Zhang et al. 2006). Plants harboring endophytes often grow faster, because e.g. fungal endophytes can produce indole-3-acetic acid, cytokines or supply of nutritional elements (e.g. nitrogen, phosphorus and sulfur) (Tan and Zou 2010; Yan et al. 2018). Moreover, phytohormones also have impact on plant metabolism, plant development and regulate the responses to the biotic and abiotic stresses (Li et al. 2012; Schmelz et al. 2016; Zhang et al. 2006).

Endophytes can enhance competitive abilities and fitness of the plants, and also entail protection against herbivores, invading plant pathogens and various abiotic stresses through plant physiology control (Gao, Dai, and Liu 2010; Hardoim et al. 2015; Saikkonen et al. 2002; Tan and Zou 2010).

In return, endophytic organisms gain nutrients and a protective refuge from the host plants (Aly, Debbab, and Proksch 2011; Saikkonen et al. 2002; Tan and Zou 2010). In addition, endophytes also benefit from vertical transmission as they are disseminated to the next generation of the host plants (Saikkonen 1998, Aly, Debbab and Proksch 2011). Plants also provide compounds that are essential for the development or self-defense of endophytic microbes (Aly et al. 2011; Strobel 2002).

Endophytic interactions are important for the plants' diversity and play an important role in the ecosystem. Biodegradation of dead or dying plant tissue is often initiated by endophytes, which start the recycling of nutrients (Aly et al. 2011; Owen and Hundley 2005; Zhang et al. 2006).

Endophytes: a potential source of new secondary metabolites

The need for new bioactive compounds for pharmaceutical and agrochemical use is growing. The development of drug resistance in microorganisms, the emergence of new dangerous viruses, the appearance of new diseases and the increase of fungal infections call for new and safe drugs (Schulz et al. 2002; Strobel and Daisy 2003).

Traditional medicinal plants are important sources of novel natural products (Yan et al. 2018). According to Li and Lou (2018), one-third of all new molecular entities approved of the FDA, originate from natural products and their derivatives. However, most medicinal plants grow very slowly and often extraction processes are time-consuming, with a low amount of active drug yield. This can lead to the over-harvesting of plants and in the worst case to an extinction of some rare plant species (Yan et al. 2018).

Current research on endophytes identified them as a high-yielding source for new bioactive compounds for agricultural, industrial and pharmaceutical applications (Aly et al. 2011; Guo et al. 2008; Zhang et al. 2006). Endophytic microorganisms

have the capabilities to produce a huge amount of secondary metabolites, which have cosmetic or therapeutic potential or already have established medicinal value (Budhiraja et al. 2013; Kaushik et al. 2014). Many of the isolated bioactive compounds of endophytic organisms have been categorized as alkaloids, steroids, terpenoids, flavonoids, phenols, isocumarins etc. (Guo et al. 2008; Owen and Hundley 2005; Schulz et al. 2002).

Endophytes from rare ecosystems or traditional medicinal plants are promising candidates for new natural products that can be discovered via bioprospecting (Martinez-Klimova, Rodríguez-Peña, and Sánchez 2017; Strobel and Daisy 2003). Endophytic microbes are known to produce natural products, which are able to limit the effect of disease-causing agents and, furthermore, kill or inhibit the pathogenic microorganisms, protozoans and viruses (Strobel and Daisy 2003).

Endophytes producing anticancer agents

Paclitaxel (Taxol) is a natural product isolated first from the bark of *Taxus brevifolia* (Pacific Yew tree) in 1971 (Guo et al. 2008; Kala and Ratajc 2012; Saha et al. 2019; Strobel et al. 2004; K. Zhao et al. 2011; Zhao et al. 2010). Taxol is a complex diterpenoid and one of the most potent drug to counteract cancer (Guo et al. 2008; Kala and Ratajc 2012; Strobel and Daisy 2003; J. Zhao et al. 2011). Paclitaxel has an antimicrotubular activity, binds to tubulin and inhibits the depolymerization of the microtubules during the cell division, finally causing cell death (Kusari, Singh, and Jayabaskaran 2014; Strobel and Daisy 2003; J. Zhao et al. 2011). The Food and Drug Administration (FDA) permitted the use of paclitaxel against breast, ovarian, prostate and lung cancer. Paclitaxel is also effective against renal, colon, cervix, gastric, pancreatic and head and neck cancers (Kala and Ratajc 2012; J. Zhao et al. 2011). *Taxus* species contain a low amount of paclitaxel, and for the extraction, the bark of these gymnosperms needs to be removed, which causes the death of the trees (Banerjee et al. 1996; Kala and Ratajc 2012; Kwak et al. 1995). In 1993, Stierle and colleagues discovered the first paclitaxel-producing fungus *Taxomyces andreanae* from *Taxus brevifolia* (Kala and Ratajc 2012; Stierle et al. 1993; J. Zhao et al. 2011). This discovery revealed that endophytes were also able to produce this

anticancer drug. Today, about 20 different genera of paclitaxel-producing endophytic fungi are known, for example *Alternaria*, *Fusarium*, *Mucor*, *Pestalotiopsis* and *Taxomyces*. These endophytes are mainly found in the species *Taxus* but also in other species like *Citrus medica* (Rutaceae), *Ginkgo biloba* (Ginkgoaceae) or *Hibiscus rosa-sinensis* (Malvaceae) (Saha et al. 2019; J. Zhao et al. 2011). *Pestalotiopsis microspora* was the first isolated taxol-producing endophyte from a non-*Taxus* species (Strobel et al. 2004). Interestingly, some of the host plants themselves have not been found to produce paclitaxel or its derivatives (J. Zhao et al. 2011).

Vincristine and vinblastine are vinca alkaloids originally extracted from *Catharanthus roseus*, belonging to the family Apocynaceae. Vinca alkaloids producing endophytes were also isolated from the phloem of the same plant. *Alternaria* sp. and *Fusarium oxysporum* had also the ability to produce vinblastine and vincristine. These two vinca alkaloids bind to the tubulin dimers, prevent polymerization and further inhibit the assembly to microtubules (Aly et al. 2011; Kala and Ratajc 2012; Saha et al. 2019; J. Zhao et al. 2011). Vinblastine and vincristine are potent anticancer drugs for the treatment of lymphoma and leukemia (Kala and Ratajc 2012).

Podophyllotoxin is a lignan from the roots of *Podophyllum* species (Berberidaceae). Podophyllotoxin prevent cell division by destabilizing the microtubules and is mainly used as antiviral compound helping to treat warts. Etoposide and teniposide, two semisynthetic glycosides of podophyllotoxin, act as topoisomerase II inhibitors and are potent anticancer drugs against lung cancer, testicular cancer and different varieties of leukemia. (Kala and Ratajc 2012; Saha et al. 2019; J. Zhao et al. 2011). Due to the increased use of podophyllotoxin and the unsuccessful agricultural production, this plant was overexploited and declared as endangered species. New sources for this valuable bioactive compound have been discovered. Endophytes not only from *Podophyllum* species but also from *Juniperus* species were isolated, which also produce podophyllotoxin. Currently, fungal culture for commercial production provide an adequate source of podophyllotoxin (Kala and Ratajc 2012; Zhao et al. 2011).

Endophytes producing antibiotics

The resistance of microorganisms to extensively used antibiotics is one of the major worldwide healthcare problems in the 21st century (Alanis 2005; Wright 2012). In 1928 the discovery of penicillin by Fleming was the start of the golden era of antibiotics. Antimicrobial agents have become an indispensable part of our medicinal care. Soon the first resistances appeared, bacterial pathogens developed different mechanisms to secure their survival. During this time, numerous antibiotics were developed including semi-synthetic and synthetically produced or modified substances. However, as a result of the increased irrational antibiotic use in humans and livestock, bacteria became resistant faster over time (Bbosa et al. 2014; Gold and Moellering 1996; Wright 2012). Endophytic microorganisms as a source of new antibiotics leads to an alternative strategy to counteract the drug resistance of human and plant pathogens (Joseph and Priya 2011; Song 2008; Yu et al. 2010).

Actinobacteria, the largest phylum of the bacteria kingdom, are important producers of a tremendous number of bioactive compounds (Dhakal et al. 2017; Lee et al. 2018). More than 65% of approved antibiotics originate from actinobacteria, and over 10.000 antimicrobial compounds were shown to be produced by the genus *Streptomyces* (Lee et al. 2018; Subramani and Aalbersberg 2012; Zotchev 2012). Since the discovery of streptomycin, chloramphenicol, tetracyclines and macrolides, *Streptomyces* species have been an outstanding source of new antimicrobial agents (Castillo et al. 2002; Janos Berdy 2005; Jose and Jebakumar 2013).

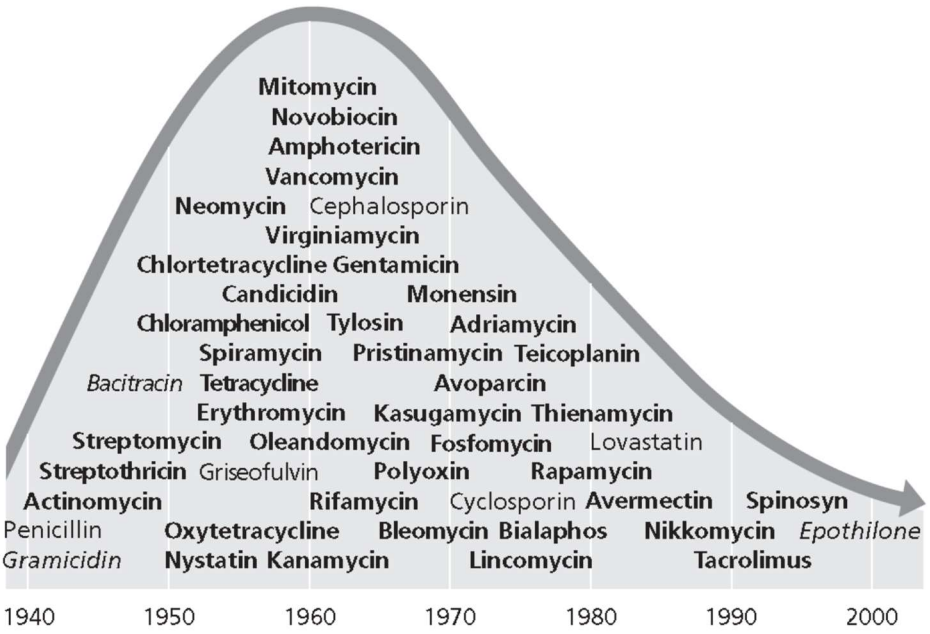

Figure 3: Timeline of the discovery of new antibiotics and other novel bioactive compounds. Bold type: actinomycete bacteria products. normal: fungal products; italic: non actinomycete bacteria products (Hopwood 2007).

Streptomyces sp. isolated from *Kennedia nigriscans* (Fabaceae), a native plant of Australia, produces novel bioactive compounds named munumbicins. Munumbicins represent a novel family of peptide antibiotics that have a broad spectrum of activity against many pathogenic fungi and bacteria. This endophytic organism was the first antimicrobial bioactive *Streptomyces* strain isolated from a woody plant (Castillo et al. 2002, 2003). In recent times, finding novel antimicrobial compounds from actinobacteria has decreased because of the rediscovery of known compounds (Jose and Jebakumar 2014). In view of that fact, the discovery of rare actinobacteria occupying special habitats in nature for the search of potential antimicrobial leads are in the scientific focus (Castillo et al. 2003; Jose and Jebakumar 2013). Currently, only a few semi-synthetic antibiotics derived from rare actinomycetes are in the clinical development or reached the market (Genilloud 2017).

Other examples of natural compounds produced by endophytes

Kusari et al. (2008) reported two natural products, the naphthodianthrone derivative hypericin and emodin, produced by endophytic fungi from stems of *Hypericum perforatum*.

Zhang et al. (1999) isolated an antidiabetic agent that acts as an insulin mimetic from an endophytic fungus from a rainforest in Africa. This small bioactive molecule activates the insulin receptor tyrosine kinase and was tested in biochemical and cellular assays (Pandey et al. 2014).

Pestacin and isopestacin, two compounds with antimicrobial and antioxidant activity, were produced by *Penicillium microspora* isolated from *Terminalia morobenis,* a plant from Papua New Guinea (Strobel and Daisy 2003).

Up to now, endophytic organisms have been isolated from hundreds of plants. A broad range of novel bioactive compounds with anticancer, antimicrobial, antioxidant and cytotoxic activities were obtained, which have potential for medicinal, industrial use or for biocontrol against pests in agriculture (Strobel and Daisy 2003; Vega et al. 2008; Zhao et al. 2010).

Tissue culture of medicinal plants using endophytic organisms as elicitor

Plant tissue culture, the technique of growing organs, tissues or single cells of plants under sterile conditions in artificial nutrient media (Kumar 2003), has become an important tool for the production of therapeutic agents. *In vitro* cultivation offers the opportunity to cultivate explants totally independent from climatic and geographical factors. Plants with a long cultivation time, which are difficult to cultivate, or plants with low yield of secondary metabolites can be efficiently cultivated by different *in vitro* techniques like cell suspension or callus culture. Furthermore, the micropropagation as well as the in vitro conservation counteracts the overexploitation of threatened and rare medicinal plants (Sarasan et al. 2006; Veeresham and Chitti 2013).

Although in vitro plantlets are cultivated in aseptic conditions, the observation of endophytic bacteria outgrowth is not uncommon. In the past, they were often considered as contaminants and were eliminated prophylactically by antibiotics.

Until recently, numerous studies indicated a positive influence of endophytes on plants cultivated *in vitro* (Goh et al. 2013; Quambusch et al. 2014).

The establishment of cell suspension cultures of various plants have been reported in several studies as an alternative source for the production of bioactive metabolites (Yue et al. 2014). Molecules of biological and non-biological origin can trigger the production of secondary metabolites in the process called elicitation (Dörnenburg and Knorr 1995). Elicitation is an effective strategy to increase the production of bioactive compounds in cell suspension cultures (Gadzovska Simic et al. 2015; Karuppusamy 2009; Salehi et al. 2019; Yukimune et al. 1996). Wang, Wu, and Mei (2001) established successfully the paclitaxel production in *Taxus chinensis* var. *mairei* with co-culture of an endophytic fungus. Another study showed a significant induction of naolide A production in root cell suspension of *Withania somnifera* with *Aspergillus terreus* 2aWF as elicitor (Kushwaha et al. 2019). Furthermore, fungal endophytes are very effective in improving the callus biomass (Tonk et al. 2016).

Endophytic elicitors can be a good approach for the enrichment of important medicinal compounds in plant cell suspensions (Pawar et al. 2011).

Process of endophyte isolation

Many bioactive substances have already been isolated from endophytes, but they are still an underused source of new bioactive secondary metabolites due to the relatively difficult isolation (Hallmann, Berg and Schulz 2006; Ryan et al. 2008). The establishment of an isolation procedure for endophytic microorganisms is a critical step. The isolation method must be gentle enough for the recovery of the native diversity of endophytic microorganisms and concurrently stringent enough to eliminate epiphytes that are found on the surface of the plant (Hallmann et al. 2006; Silvani et al. 2008). For an effective isolation, separate procedures should be adapted for each plant tissue type. In general, the isolation process consists of the sterilization of the plant tissue surface, the maceration of the respective plant tissue and subsequent streaking the macerate onto nutrient media.

The plant surface disinfection is carried out with an appropriate chemical, which should kill the epiphytes on the plant surface without harming the endophytic microorganisms inside the plant. Nevertheless, the sterilization conditions necessary to eliminate the last epiphytic organism on the surface may result in the sterilization agent penetrating the internal tissues and thus harming some endophytes.

The first step of the sterilization is washing the plant under running tap water to remove contaminants such as soil, debris and epiphytes from the surface (Hallmann, Berg and Schulz 2006; Lodewyckx et al. 2010). Then, plant tissues are immersed in disinfecting agents under sterile conditions. Commonly used disinfectants are ethanol (EtOH), sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2). Mercuric chloride is also a very effective sterilization agent, but because of the safety issues, should only be used if other disinfectants fail (Hallmann, Berg and Schulz 2006; Larran et al. 2002). To enhance the effectiveness of the surface sterilization, a detergent such as Tween or Trition X-100 can be added to the sterilizing agent to lower the surface tension (Hallmann, Berg and Schulz 2006; Oyebanji et al. 2009). At the end of each treatment, the plant must be rinsed in sterile water to remove traces of the sterilizing agents.

Two methods are common to validate the effectiveness of the surface sterilization protocol. The first is the imprinting of the disinfected plant tissue onto growth media. The second comprises streaking out aliquots of the last washing water onto the nutrient media (Hallmann, Berg and Schulz 2006; McInroy and Kloepper 1994).

The best method to isolate the endophytes is maceration of surface sterilized plant tissue. By grinding the plant, all the endophytes in each tissue and niche of the plant are released. Maceration enables the coverage of a broad spectrum of cultivable endophytic organisms. Maceration can be conducted manually with a mortar or mechanically with a blender or a homogenizer depending on thickness and rigidity of the plant material and on sample size (Hallmann, Berg and Schulz 2006). Precellys®24 is an efficient bead-beating homogenizer. The high speed and the 3-dimensional figure-8 multidirectional motion in combination with Precellys™ lysis kits prefilled with beads of different sizes and materials provide

efficient maceration of biological samples (Verollet 2008). For each plant and plant tissue, the intensity and duration of maceration must be determined empirically. Plant bioactive substances and enzymes are released during maceration and can kill or inactivate endophytes. The heat generated during homogenization promotes the latter processes, so adequate cooling of the plant material must be ensured to prevent the increase of temperature (Hallmann, Berg and Schulz 2006).

The choice of appropriate growth media is an important factor which determines the isolation of tissue specific endophytic microorganisms related to their amount and diversity (Eevers et al. 2015; Hallmann, Berg and Schulz 2006). Only a minor fraction of 0.001% to 1% of the endophytes are cultivatable with current methods (Alain and Querellou 2009; Eevers et al. 2015). Non-selective culture media promote a plethora of different microorganisms present in the plant macerate (Hallmann, Berg and Schulz 2006; Lagier et al. 2015). A typical growth medium for a broad range of bacteria is TSA, and for fungi PDA (Hallmann, Berg and Schulz 2006). The isolation of rare endophytic microorganisms, e.g. actinobacteria, requires selective isolation media that give these microorganisms advantage over competing organisms. Specialized growth media containing proteins, amino acids, humic acid and other nitrogen sources as well as carbon containing sources for example starch or chitin can be used for isolation of rare actinomycetes. Further ingredients for the preferential isolation of actinobacteria are e.g. L-arginine, L-asparagine, B-vitamins and trace salts (Hayakawa and Nonomura 1987; Kumar and Jadeja 2016).

Antibiotics, fungicides and specific nutrients supplemented to culture media can inhibit the growth of various bacterial and fungal organisms (Hallmann, Berg and Schulz 2006; Kumar and Jadeja 2016; Lagier et al. 2015). Actinomycetes and fungi can grow together on the same nutrient media, therefore addition of antibiotics that suppress fungi is useful in the isolation of these bacteria. Nystatin and cycloheximide were recommended as antifungal agents (Kumar and Jadeja 2016). If fungal endophytes need to be isolated, such antibiotics against bacteria as trimethoprim, gentamicin, kanamycin and chlortetracycline can be used (Hayakawa 2008; Kumar and Jadeja 2016).

After successful isolation and cultivation, endophytic bacteria can be compared and identified using 16S rRNA gene sequencing, a popular used method for comparing bacterial phylogeny (Tremblay et al. 2015).

Identification of endophytes by 16S rRNA gene sequencing

Microbial systematics has constantly changed over time, resulting in numerous re-classifications. Bacterial taxa have been renamed, new taxa created or dissected. Classical identification methods based on deoxyribonucleic acid (DNA) guanine and cytosine content (G + C) or biochemical profiles are useful to differentiate taxa. Identification of phylogenetic relationships and classification at and below the species level needs more precise methods, such as DNA-DNA hybridization (Ludwig and Klenk 2001).

Sequencing and comparison of the small subunit ribosomal RNA (16S rRNA) gene sequences has become the most common way for classification of bacteria. The 16S rRNA is a ubiquitous gene, has a high information content and is well conserved (Janda and Abbott 2007; Lane et al. 1985).

The 16S-rRNA gene, an approximately 1500 base pair long, is highly conserved within a species and can be used for taxonomical classification and phylogenetic analysis of bacteria (Petti 2007; Stackebrandt and Goebel 1994; Woo et al. 2001). Many species have just a single copy of the 16S-rRNA gene but in some bacteria the number of copies rise up to 15 (Kembel et al. 2012; Lee, Bussema, and Schmidt 2009; Rastogi et al. 2009). 16S-rRNA gene contains conserved, semi-conserved and hypervariable or non-conserved regions (Gray, Sankoff, and Cedergren 1984). Nine highly variable regions in the 16S-rRNA gene are unique for bacterial species and allow identification of closely related organisms (Chakravorty et al. 2007; Peer 1996; Petti 2007).

Despite of taxonomic research, 16S-rRNA gene sequencing is also used for the discovery of new pathogens in clinical microbiology and allows the identification of non-cultured bacteria.

The polymerase chain reaction (PCR) represents an essential method for effective studies on the16S-rRNA genes (Kennedy et al. 2014; Lantz, Hahn-

Hägerdal, and Rådström 1994). For analysis of the 16S-rRNA gene, bacterial genomic DNA is used as a template for PCR amplification and sequencing approaches (Clarridge III 2004).

16S-rRNA gene-based analysis is a powerful identification method for microbial communities, however, this molecular approach has also some limitations.

First, the determination of species identity is in some taxa often impossible, due to variation within the 16s-rRNA gene for example in the *Enterobacteriaceae* (Mignard and Flandrois 2006; Mollet, Drancourt, and Raoult 1997). Furthermore, due to the low polymorphic nature the application of the 16S-rRNA gene sequence is inefficient for the identification of closely related species of *Acinetobacter* (Scola et al. 2006).

Good sequence quality is the second limitation, which is essential for the interpretation (Mignard and Flandrois 2006).

Phylogenetic analyses, the study of relationships between individuals in an evolutionary context, was revolutionized by the development of new molecular technologies since the 1980s. Especially the phylogenetic study of microorganisms profited from molecular approaches. Today, a combination of phylogenetic studies based on gene sequence information and morphological characteristics are state of the art describing new species (Fitz-Gibbon and House 1999; Pagel 1999). A set of sequences of individuals are compared to calculate their relationship by the use of different methods and models (Anisimova et al. 2013). The phylogenetic content resulting from such sequence alignments can be visualized by graphical methods which creates an illustration of evolution by tree- net- or star-like geometry (Strimmer and von Haeseler 1997).

Aims of study

Leontopodium nivale subsp. *alpinum*, one of the best-known wild flowers, resides in sub-alpine and alpine regions of the Austrian Alps. The natural habitat of this plant species in the central European Alps presents a fragile ecosystem with fragmented biotopes in extreme microclimatic conditions. Adaptation strategies of alpine plants in this environment are diverse and often depend on symbiotic associations with endophytic microorganisms. Alpine plants and in general, alpine ecosystems are promising sources for novel bioactive metabolites. Bioprospecting endophytes to find new drugs for the treatment of human diseases, livestock and plants are thus of great interest.

The aim of this study was to isolate endophytic microorganisms from the leaves, rhizomes and roots of native alpine *Leontopodium nivale* subsp. *alpinum* originating from the Rax plateau. Isolates were cultivated and the identification with 16S-rRNA gene sequencing was started. The endophytes of these alpine plant may have potential to produce novel secondary metabolites.

The second aim of this work was the establishment of *in vitro* and callus cultures of Edelweiss as a preliminary work towards the enhancement of secondary metabolite production in plant cell suspension cultures.

Materials and methods

Plant collection

Collection of plants from the medicinal plant garden

Plants of *Leontopodium nivale* subsp. *alpinum* var. *helvetica* were harvested for optimization procedures from the medicinal plant garden of the Department of Pharmacognosy, University of Vienna, Austria. Plants were sealed in plastic bags and stored at 4 °C in a fridge until surface sterilization.

Collection of native alpine plants

Leontopodium nivale subsp. *alpinum* is listed on the Plant Protection Regulation of Lower Austria (Appendix A). The Office of the Provincial Government of Lower Austria, Department Nature Conservation (Amt der NÖ Landesregierung, Abteilung Naturschutz) granted a collector´s permission to Franz Tod, (Botanical Garden, University Vienna) (Appendix B). He accompanied the field collection trip.

Each plant individual was harvested as a whole including roots, rhizosphere soil and all rhizomes and leave rosettes. Plants were sealed in plastic bags and stored on ice in a freezer box for transportation to the laboratory. Plants were kept at 4°C and processed within 48 hours after harvest. For later analyses, also soil samples were collected from each collection site. The soil was sieved to separate stones and organic materials before storing it in 50 ml Falcon (Sarstedt, Nümbrecht, Germany) tubes at -80°C.

Surface sterilization

Plant samples were thoroughly rinsed under a gentle flow of running tap water to remove adhering soil and debris (Figure 4). Plants were separated into the tissue’s roots, rhizomes, leaves and stems and placed in 50 ml Falcon tubes and stored in the refrigerator at 4 °C till sterilization process.

Figure 4: Alpine plant sample rinsed under running tap water

Plant tissues were surface sterilized under sterile conditions in a laminar flow hood with autoclaved deionized water (dH2O) supplemented with different concentrations of surface sterilization agents and with or without 0,1 % Tween-20 (Sigma Aldrich, St. Louis, Missouri, USA) by continuously shaking. Subsequently, plant tissues were rinsed with sterile water to remove traces of sterilant, then immersed in sterile water.

Plant surface sterilization included variation of the following parameters: sterilization agent, with and without tween, and with and without magnetic stirrer, time of exposure and different washing conditions (Tables 4 and 5).

Explants cultivation

The basis of the disinfected rosettes of Edelweiss was fixed with forceps and the leaves were gently removed with another forceps until the apical meristem appeared. The explants were aseptically placed in eprouvettes (Figure 5) containing 13 ml of autoclaved agar-solidified MS basal media at a pH 5,7 and closed with Magenta™ 2-way caps (Sigma, Merck KGaA, Darmstadt, Germany).

Figure 5: Cultivar “Helvetia” in eprouvettes closed with Magenta-2-way caps.

For callus induction, leaf pieces of approximately 0,5 cm^2 were excised from sterilized healthy appearing leaves in the laminar flow hood. They were then transferred to eprouvettes containing 13 ml of autoclaved MS media with 5 µl 2,4-dichlorophenoxyacetic acid (2,4-D) and closed with Magenta™ 2-way caps. The pH was adjusted on 5,7 by using 0,1 N sodium hydroxide or 0,1N hydrochloric acid.

All explants were cultivated in a growth chamber at 25±1 °C with a photon flux density of 40 µM m^{-2} s^{-1}, 50 % relative humidity and 16 hours photoperiod.

Maceration

Maceration of plant samples was performed with a Precellys® 24 homogenizer (Bertin Technologies, France) based on bead beating technology (Figure 6).
No literature or information from the manufacturer of the Precellys® 24 tissue homogenizer was recommended for isolation of endophytes. Therefore, a maceration protocol was developed. Maceration is a major process for endophyte isolation. For the emergence of endophytes from inside the plant tissue, the plant cells must be ground into optimal fragments. Several pilot tests for different bead tubes and parameter settings (volumes, agitation duration and agitation speed)

for the maceration using a Precellys® 24 tissue homogenizer were conducted and evaluated macroscopically and microscopically. Plant tissue were cut in small pieces and transferred in 2 ml Precellys® tubes containing 1 ml 20% glycerol. Samples were stored on ice before maceration and thereafter.

Figure 6: Precellys® 24 homogenizer

Isolation media

Six different media were chosen to cover combined a broad spectrum of bacteria and fungi. Four from these isolation media types were additionally combined with two different antibiotics (Table 1). Potato dextrose agar (PDA) and tryptic soy agar (TSA) were used in all pilot test as general growth media for fungi (PDA) and bacteria (TSA) to verify the sterility of the different methods.

Growth media were prepared in 1 l Schott Duran® flasks. Ingredients listed in Table 1 were dissolved in deionized and distilled water by using a magnetic stirrer (M3-D, Agro Lab, Germany). Afterwards, nutrient media were autoclaved at a temperature of 121 °C and a pressure of 1 bar for 20 minutes.

Aliquoted antibiotics were thawed on ice and then added to autoclaved and cooled down media, which had a temperature shortly below the solidification point

of the agar (approximately 50-40°C). Subsequently the flask was agitated gently by hand to efficiently disperse the antibiotics.
In a sterile bench, media were poured into petri dishes with a diameter of 9 cm. While cooling down to room temperature the petri dishes were open to prevent condensation of the steam on the lids and solid media. Selected media were stored at room temperature in the dark, those with the addition of antibiotics were stored at 4 °C in a fridge until use.

Antibiotics as selectors

Nystatin is a pore-forming polyene macrolide antibiotic and binds ergosterol, present in the fungal cell membrane. In selective media, nystatin was used for suppression of fungal growth (Santos et al. 2017). A 10 mg/ml stock solution was prepared by dissolving nystatin (Sigma Aldrich®, St. Louis, Missouri, USA; N3503-3MU) in dimethyl sulfoxide (DMSO). Stock solution were aliquoted in 1,5 ml Eppendorf tubes and were stored at -20°C until use. In order to achieve a final concentration of 20 µg/ml of nystatin in isolation media, 2 ml of the stock solution were added to 1 l of medium.

Cycloheximide is a protein biosynthesis inhibitor by blocking the translational elongation in eukaryotic cells. Cycloheximide suppresses the growth of yeast and saprophytic molds (Schneider-poetsch et al. 2010). A stock solution containing 40 mg/ml cycloheximide (Sigma Aldrich®, St. Louis, Missouri, USA; 01810-1G) was prepared by dissolving cycloheximide in ethanol. Afterwards, the solution was sterile-filtered (Minisart SRP25, Satorius, Göttingen, Germany) and immediately aliquoted in 1,5 ml Eppendorf tubes and was stored at -20°C until use. The final concentration in isolation media was 20 µg/ml. This was achieved by adding 0,5 ml of the stock solution per liter of media.

Table 1: Composition of nutrient media per one liter for isolation of microorganisms

Ingredients (g/l)	GAC agar	HV agar	ISP2 agar	King´s B agar	PDA agar	SNA agar	TSA agar
Yeast extract	-	-	4,0	-	-	-	-
Malt extract	-	-	10,0	-	-	-	-
Dextrose	-	-	4,0	-	20,0	-	-
Glucose	2,0	-	-	-	-	0,2	-
Glycerol	-	-	-	10 ml	-	-	-
Casein peptone	-	-	-	-	-	-	15,0
Pepton	-	-	-	20,0	-	-	-
Soy peptone	-	-	-	-	-	-	5,0
Humic acid	-	1,0	-	-	-	-	-
Potato starch*	-	-	-	-	4,0	-	-
Sucrose	-	-	-	-	-	0,2	-
L-Asparagine	1,0	-	-	-	-	-	-
Humic acid	-	-	-	-	-	-	-
K2HPO4*3H2O	0,4	-	-	1,5	-	1,0	-
CaCO3	-	0,02	-	-	-	-	-
NaCl	-	-	-	-	-	-	5,0
KCl	0,3	1,7	-	-	-	0,5	-
KNO3	-	-	-	-	-	1,0	-
NaH2PO4	-	0,5	-	-	-	-	-
MgSO4*7H2O	0,3	0,5	-	1,5	-	0,5	-
FeSO4*7H2O	0,01	0,01	-	-	-	-	-
CuSO4*5H2O	0,001	-	-	-	-	-	-
ZnSO4*7H2O	0,001	-	-	-	-	-	-
MnSO4*7H2O	0,001	-	-	-	-	-	-
B-vitamins**	-	+	-	-	-	-	-
Leaf extract		+					
Nystatin	+	+	+	+			
Cyclohexamide	+	+	+	+			
Agar	20,0	18,0	20,0	15,0	15,0	12,0	15,0
pH	7,4	7,2	7,2	7,2	5,6	5,4	7,3

*approx. 200 g of infusion from potatoes

**0,25 mg L-1 biotin and 0,5 mg L-1 of thiamine HCl, riboflavin, niacin, pyridoxin HCl, inositol, Ca-pantothenate, p-aminobenzoic acid

Humic acid vitamin B (HV) media was supplemented with Leontopodium nivale subsp. alpinum leaf extract

According to Eevers et al. 2015, the number of cultivable endophytic bacteria can be increased by adding a plant extract to the selective medium.

Leaves of Edelweiss were harvested from the medicinal plants garden (Pharmaceutical Sciences, Althanstraße 14, 1090 Vienna) and stored at -20 °C overnight. Plant material was lyophilized and weighted. 40 g of dried leaves were mixed in a beaker with 1,5 l of deionized and distilled water. Afterwards the mixture was blended with a hand blender and was filtered with a fluted filter. The leaf extract was aliquoted into four 1 l Schott Duran flasks, the remaining ingredients of the media (Table 1) were added and filled up with dH2O. The HV media with leaf extract was autoclaved at 121 °C, 1 bar for 40 minutes.

Preparation of serial dilutions and plating macerates

100 µl of each tissue macerate was serially diluted in 900 µl dH2O. Four dilution series from the original macerate 10^0 down to 10^{-3} were prepared. 100 µl aliquots of the macerate of each dilution were plated on to six different solid isolation media. In total 360 plates were prepared.

5 plants x 3 tissues x 4 dilutions x 6 media = 360 plates

All plates were stored in an incubator at room temperature to avoid rapid growth of endophytes (Figure 7).

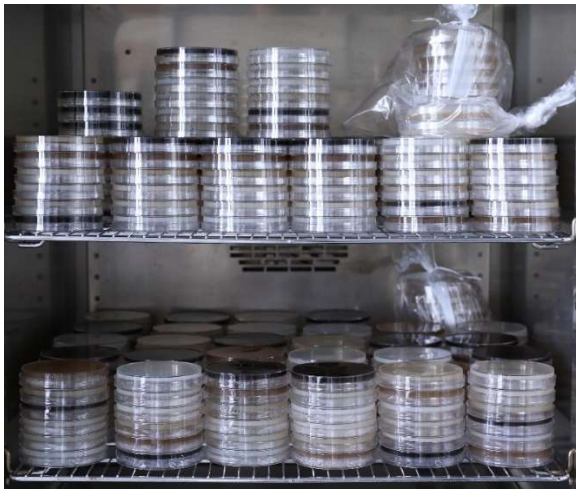

Figure 7: Plates with plant macerates in different dilution on different solid isolation media.

Petri dishes were daily inspected for one month and twice a week for the following next two months. Colonies of bacterial and fungal isolates were characterized by the following features: shape, color, size, surface, texture and opacity. Each isolate was recorded in a excel sheet by Dr. Martina Oberhofer (Appendix C).
Selected colonies were picked with an inoculating loop under sterile conditions and inoculated onto a new TSA plate by the fractionated streak out method to dilute the number of microorganisms and to assure that the appearing colony is just from one organism. Afterwards a single pure colony was picked and transferred onto a new TSA plate and incubated at room temperature. If bacteria didn´t grow on TSA media, the same colony was cultivated onto a new agar plate with the same media of the isolation plate. Resulting pure cultures were used for production of liquid cultures and in some cases spore isolation plates.
Isolation of fungal microorganisms was done by Dr. Martina Oberhofer. Therefore, from now on the term endophytes in respect to this Diploma Thesis will only refer to endophytic bacteria.

Liquid cultures

Endophytes without visible sporulation were cultivated in liquid media. Therefore, single colonies were transferred with a sterile tooth pick in a 15 ml round bottom tube containing 3 ml tryptic soy broth (TSB). Liquid cultures were incubated under continuously shaking at 28 °C at 200 rpm until growth were observed. 800 µl of liquid culture were diluted 1:1 with 100 % glycerol in cryotubes. Duplicates of each liquid culture were stored at - 80 °C. The remaining part of liquid cultures were stored at 4 °C for further use.
After one week without growth the cultivation was repeated in 3 ml twofold yeast extract/tryptone (2xYT) or 3 ml 20 % TSB respectively.

Preparation of sporulating plates for Actinomycetes

Purified isolates, which had the ability to produce spores on isolation plates were newly streaked out onto an agar plate. After sporulating, the spores were harvested under sterile conditions and stored at -80 °C. Approximately 5 ml 50 % glycerol was pipetted onto plates with sporulating bacteria and carefully dispersed with a spatula. The suspension was transferred to sterile 5 ml syringes containing cotton wool as filter material. Mycelia and other cell material of bacteria

remained in the cotton wool. 1 ml of the pure spore suspension was transferred to cryotubes and stored with a duplicate at -80 °C.

Endophytes, which were not sporulating as well as not cultivable in liquid media, were scraped off from the isolation plate by adding 1 ml 50% glycerol before transfer by a spatula. The suspension was subsequently pipetted in 2 ml cryotubes containing 800 µl 50 % glycerol and stored at -80 °C.

DNA Extraction

DNA extraction was performed using a method modified from Queipo-Ortuño et al. (2008). DNA from isolates were extracted by boiling.
1 ml of liquid culture media with growing bacteria were transferred into sterile Eppendorf tubes and centrifuged at 14.000 revolutions per minute (rpm) for 5 minutes to form a cell pellet. The supernatant was removed, and the pellets were stored at -20°C for DNA extraction. For DNA extraction, 1 µl of the pellet was resuspended in 250 µl sterile deionized water under sterile conditions in a 2 ml Eppendorf tube. In each cap of Eppendorf tubes, a hole was made to compensate the pressure during the boiling process and were covered with a paper towel. Tubes were placed in a heating block (Accu Block™, Labnet International, New Jersey, USA) and incubated for cell lysis at 100°C for 12 minutes. Afterwards the tubes were cooled on ice then centrifuged for 3 minutes at 14.000 rpm at room temperature. The supernatant containing the bacterial DNA was transferred to a clean tube and was used immediately for amplification of 16S-rRNA gene fragment.

Amplification of 16S rRNA gene fragment using bacterial endophytes

Standard forward (27F: 5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse (1492R: 5′-TACGGY TACCTTGTTACGACTT-3′) primers were used for polymerase chain reaction (PCR) amplification of 16S-rRNA gene fragments.

Table 2: PCR master mix for the amplification of 16S rRNA gene fragment

Taq buffer (200 mM Tris HCl, 500 mM KCl, 50 mM $MgCl_2$)	4 µl
dNTPs (deoxy nucleotide triphosphates)	0,5 µl
Forward primer (10 mM)	1 µl
Reverse primer (10 mM)	1 µl
Taq DNA polymerase	1 µl
Pfu DNA polymerase	0,34 µl
DMSO 10 %	2 µl
Sterile deionized water	29,2 µl

39 µl of the PCR master mix (Table 2) and 1 µl of genomic DNA were transferred to strip tubes or a 96-well plate (Sarstedt, Nümbrecht, Germany). The negative control was performed with 1 µl sterile dH_2O and 39 µl of the PCR master mix. The PCR amplification was performed with an Eppendorf Mastercycler® Nexus X2 (Eppendorf AG, Hamburg, Germany) or a Biometra T3 Thermocycler (Analytik Jena AG, Jena, Germany). PCR condition were as followed: initial denaturation at 95°C for 2 minutes, then 30 cycles of denaturation at 95 °C for 30 seconds, followed by annealing at 60 °C for 30 seconds and extension at 72°C for 3 minutes. A final elongation was conducted at 72 °C for 5 minutes followed by a 4 °C hold temperature after completion of the PCR program.

Gel electrophoresis

PCR was checked by gel electrophoresis. DNA fragments are separated by applying an electric current, negative charged DNA molecules are moving to the anode (positive electrode). Short DNA fragments migrate faster through an agarose matrix than longer DNA fragments, because they are moving more easily through the pores of the gel matrix (Lee et al. 2012). The gel consisted of 0,8 % agarose in 500 ml Tris/Borate/EDTA (TBE) buffer and 25 µl of GelRed™ DNA stain (New England Biolabs GmbH, Frankfurt am Main, Germany), to dye nucleic acid by intercalation. 3 µl of the PCR product were mixed with 1 µl blue loading dye (New England Biolabs GmbH, Frankfurt am Main, Germany) and loaded into the gel slots. In the first slot, the 1 kbp DNA ladder (New England Biolabs GmbH, Frankfurt am Main, Germany) was applied, to determine the size of DNA fragments. In the last gel well the negative control (H2O used in PCR

reaction) was added. Electrophoresis was performed for approximately 25 minutes at 100 V electric current.

PCR product purification

The PCR products were purified using the DNA Clean & Concentrator Kit™ (Zymo Research, city, Country of headquarter). 33 µl of each PCR product were mixed with 165 µl binding buffer in 1,5 ml microcentrifuge tubes by vortexing. The mixture was transferred to a provided Zymo-Spin™ Column in a Collection tube. Tubes were centrifuged at 14.000 rpm for 30 seconds. After discarding the flow-through, 200 µl of DNA wash buffer was added to the column. Afterwards, the columns were centrifuged at 14.000 rpm for 30 seconds. The washing step was repeated once more with the same volume. The flow-through was discarded and columns were centrifuged to dry the column matrix containing the bound DNA at 14.000 rpm for 30 seconds. 40 µl of DNA elution buffer was directly added to the column matrix and incubated at room temperature for 5 minutes. The columns were transferred into 1,5 ml microcentrifuge tubes and purified DNA eluted by centrifuging at 14.000 rpm for 30 seconds.

Nanodrop measurement

The concentration of purified PCR products was determined using the Thermo Scientific™ NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). According to instructions by Eurofins Genomic (Company) the minimum concentration for sequencing is 10 ng/µl DNA.

Sequencing

17 µl of purified PCR products were mixed with 2 µl forward and 2 µl reverse primer in coded tubes of the Mix2SeqKit provided by Eurofins Genomic (Eurofins Genomics AT GmbH, Austria). The second method was to transfer unpurified PCR products with a concentration of minimum 10 ng/µl in a 96-well plate from Eurofins Genomics. The samples were purified and sequenced by the company using the Sanger sequencing method.

The software Geneious Prime (Geneious Prime® 2019.2.1) was used for manual editing of sequencing results. Low quality regions of the raw sequence data were

trimmed from 3’ and 5’ end of the both sequenced fragments. Afterwards the sequences were aligned and assembled.

The consensus sequences were entered in the nucleotide BLAST (Basich Local Alignment Search Tool) database from NCBI (National Center for Biotechnology Information, Maryland, USA) and were blasted to compare with all sequences in this database.

Results

Plants of *Leontopodium nivale* subsp. *alpinum* (Cass.) variation *helvetica* were used for experiments to establish optimized plant surface sterilization and maceration protocols. Plants were grown in the medicinal plants garden of the Department of Pharmacognosy (University of Vienna).

Five alpine plant individuals of *Leontopodium nivale* ssp. *alpinum* (Edelweiss) were collected from their native habitat at the Rax plateau, a mountain along the border between Styria and Lower Austria (Figures 8 and 9).

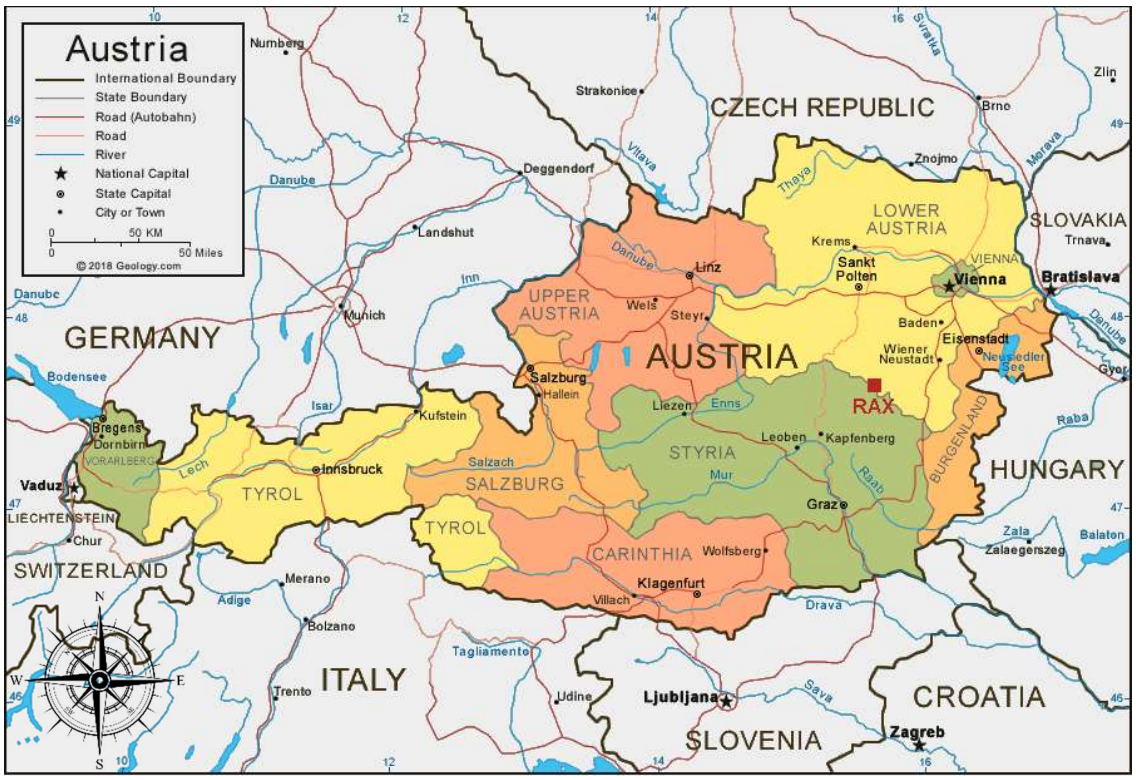

Figure 8: Map of Austria with the location of the Mount Rax marked with a red rectangle. (https://geology.com/world/austria-satellite-image.shtml)

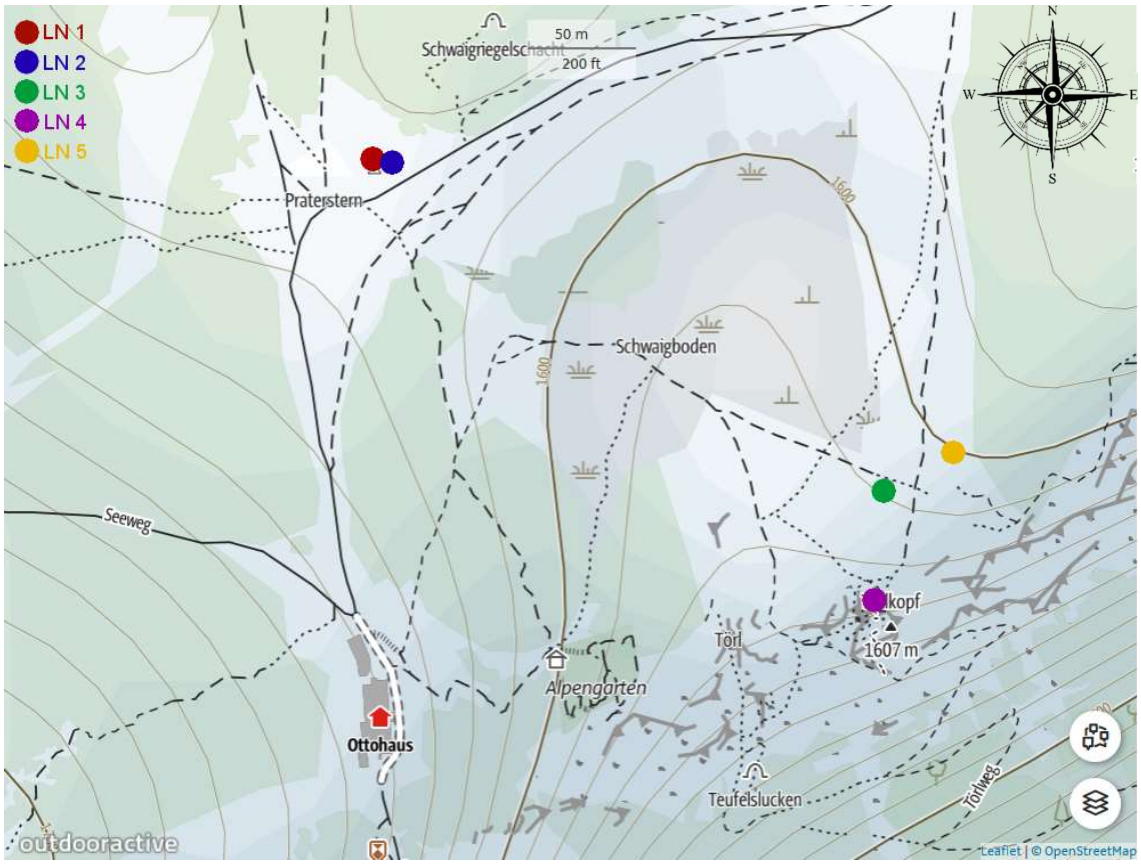

Figure 9: Closeup on the collection sites of the five *Leontopodium nivale* subsp. *alpinum* plant individuals (LN1-5) that were collected at Mount Rax between Praterstern and the Schwaigboden in 2016.
(https://www.outdooractive.com/de/routeplanner)

Plants were chosen to represent heterogeneous biotope characteristics, which may result in a higher diversity of their endophytic community to increase the possibility of finding rare endophytic microorganisms. Environmental parameters included vegetative or generative life stages of the plant, soil depth, inclination and co-occurring plant species. Around each plant, we delimited a square of 1 m^2 and evaluated all co-occurring species that were within its limits. Vegetation analysis was done by Dr. Oberhofer and Franz Tod. Global position system (GPS) data were recorded for all individual plants, detailed location information and soil condition are listed in Table 3.

Table 3: Indicator values of plant samples from *Leontopodium nivale* subsp. *alpinum*

Plant sample ID	Name of location	Coordinates (N/E)	Altitude (m.a.s.l.)	Inclination	Description of biotop
LN 1	Praterstern	N47°42'57.79'' E15°48'43.52''	1617 m	14°	Soil depth up to 10 cm, in one point the bedrock emerged trough Humus layer, no stone content Deeply rooted
LN 2	Praterstern	N47°42'57.74'' E15°45'44.06''	1617 m	12,4°	Soil depth 0–10 cm Shallow humus layer Deeply rooted, partly stones in humus layer
LN 3	Between Otto-Schutzhaus and Törlweg	N47°42'51.23'' E15°45'57.29''	1578 m	2,7°	Soil depth partly deeper than 20 cm Humus layer with moderate roots No stone content Well-aerated soil Many grass roots, fine grained soil, deep root growth
LN 4	Between Otto-Schutzhaus and Törlweg	N47°42'49.66'' E15°45'57.08''	1564 m	7,3°	Very exposed area Shallow humus layer (1–8 cm) – mostly in cracks Bedrock visible in the entire biotope Heavy surface erosion due to rainwater – topsoil partly removed Humus layer and roots exposed, thus strong heating effect of remaining humus Rooting zone exposed to direct sunlight and desiccation of soil Dense roots between stone content and bedrock Herbivore and trampling damage, Possibly fungal disease – yellowed leaves and black spots (appx. 10%)
LN 5	Between Otto-Schutzhaus and Törlweg	N47°42'52.21'' E15°45'59.18''	1674 m	13°	Very heterogenous depth (6–20 cm) Indication of higher soil water content by the presence of a macro-fungi Moderate humus layer partly with bedrock 10–15% shade

Optimization of surface sterilization

The efficacy of the surface sterilization process is significant for the isolation of endophytes. The optimization of surface sterilization to maintain endophytic microbial diversity and to remove microorganisms adhering to the plant surface was essential.

Various sterilization protocols were tested to find the best compromise between the requirements of endophyte isolation and tissue culture.

Ethanol (EtOH), sodium hypochlorite (NaOCl) and mercury (II) chloride ($HgCl_2$) were used for surface disinfection of the plant tissues. Tween 80 was used in some tests to increase the wettability of plant tissues, because Edelweiss aboveground tissues have dense trichomes. It was expected that above- and below ground tissues require different optimal treatments, because of the higher contamination of rhizomes and roots, hence plant material was separated in above- and below ground tissues.

Plant surface sterilization included variation of the sterilization agent with and without Tween, as well as with and without magnetic stirrer, time of exposure and different washing conditions (Tables 4 and 5).

In two protocols, plants were stirred in the sterilization agent continuously using a magnetic stirrer during the process of sterilization, which had a harmful effect on plant tissues. Leaf damage was observed when ethanol was used as sterilization agent.

After surface sterilization, the success of each method was either evaluated by plating washing water (wash) onto tryptic soy agar (TSA) and potato dextrose agar (PDA) media to test for absence of contaminants. Moreover, an imprint from each plant sample was made on both media to evaluate the efficacy of the surface sterilization protocols. Another method of evaluation was to plate approximately 0,5 cm^2 pieces of surface-sterilized plant onto both agar media (iso).

Table. 4: Surface sterilization experiments of aboveground tissue

Number. of sterilization protocol (No.)	Sterilization agent (percentage)	Time of exposure (min)	Addition of 0,1% tween	Usage of magnetic stirrer	Washing water (ml)	Washing duration (min)	Evaluation of sterilization	Contamination washing water (colonies)		Contamination imprint (colonies)	
								TSA	PDA	TSA	PDA
1	5 % NaOCl	1	+	+	200 200	1x 0,1 3x 10	Iso	Not evaluated (NE)	NE	NE	NE
2	5 % NaOCl	2	+	+	200 200	0,1 3x 10	Iso	NE	NE	NE	NE
3	3 % NaOCl	10	+	-	200 200	0,1 3x 10	Iso	NE	NE	NE	NE
4	7 % NaOCl	1	+	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
5	0,1 % $HgCl_2$	10	+	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
6	0,1 % $HgCl_2$	5	+	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
7	0,05 % $HgCl_2$	10	+	-	200 300	Quick 3x 10	Iso	NE	NE	NE	NE
8	5 % NaOCl	2,5	+	-	200 200	Quick 3x 5	Iso	NE	NE	NE	NE

9	0,1 % $HgCl_2$	3	+	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
10	0,1 % $HgCl_2$	5	+	-	200 200	Quick 3x 10	Wash, I	0	0	0	0
11	0,1 % $HgCl_2$	3	+	-	200 200	Quick 3x 10	Wash, I	+++	0	3	0
12	3% NaOCl	10	+	-	200 200	Quick 3x 10	Wash, I	2	0	14	1
13	2 % NaOCl	10	+	-	200 200	Quick 3x 10	Wash, I	0	0	0	0
14	0,1 % $HgCl_2$	4	+	-	200 200	Quick 3x 10	Wash, I	NE	NE	NE	NE
15	0,1 % $HgCl_2$	6	+	-	200 200	Quick 3x 10	Wash, I	2	0	0	

Table. 5: Surface sterilization experiments of belowground tissue

Number. of sterilization protocol (No.)	Sterilization agent (percentage)	Time of exposure (min)	Addition of 0,1% tween	Usage of magnetic stirrer	Washing water (ml)	Washing duration (min)	Evaluation of sterilization	Contamination washing water (colonies)		Contamination imprint (colonies)	
								TSA	PDA	TSA	PDA
16	5 % NaOCl	6	+	+	200 200	Quick 3x 10	Iso	Not evaluated (NE)	NE	NE	NE
17	7 % NaOCl	6	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
18	70 % EtOH 5 % NaOCl 70 % EtOH	1 4 1	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
19	70 % EtOH 7 % NaOCl 70 % EtOH	1 4 1	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
20	70 % EtOH 7 % NaOCl 70 % EtOH	1 4 1	-	-	200	1	Iso	NE	NE	NE	NE
21	0,1 % $HgCl_2$	10	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
22	0,1 % $HgCl_2$	7	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE

23	0,05 % $HgCl_2$	10	+	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
24	0,1 % $HgCl_2$	5	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
25	7 % NaOCl	6	+	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
26	0,05 % $HgCl_2$	3	-	-	200 200	Quick 3x 10	Iso	NE	NE	NE	NE
27	0,1 % $HgCl_2$	5	+	-	200 200	Quick 3x 10	Wash, I	+++	1	2	0
28	0,1 % $HgCl_2$	3	+	-	200 200	Quick 3x 10	Wash, I	+++	1	2	0
29	0,1 % $HgCl_2$	10	+	-	200 200	Quick 3x 10	Wash, I	0	0	0	0
30	0,1 % $HgCl_2$	7	+	-	200 200	Quick 3x 10	Wash, I	NE	NE	NE	NE
31	5 % NaOCl	6	+	-	200 200	Quick 3x 10	Wash, I	43	19	7	20
32	7 % NaOCl	6	+	-	200 200	Quick 3x 10	Wash, I	2	0	3	0

The most efficient sterilization protocol for above ground tissue was No. 10.
No surface contamination of below ground tissue was observed with sterilization protocol No. 29. These sterilization protocols were used for all further experiments.
The sterilization agent sodium hypochlorite damaged the plant tissues, especially with prolonged exposure time. This was also observed at low concentration of sodium hypochlorite.
Sterilization protocols No. 1 to 9 and 16 to 26 were excluded from the evaluation (see Discussion). Furthermore, protocols 14 and 30 were not evaluated further, because of contamination.

Optimized sterilization protocol for plant tissue culture

The sterilization protocols No 10, 11 and 12 were further evaluated to find the best surface sterilization for plant tissue culture, based on the percentage of survival of explants on MS basal media (MSØ) (Murashige and Skoog 1962) (Table 6).

Table. 6: Cultivation of *Leontopodium nivale* subsp. *alpinum* variation Helvetia on MSØ

Number of sterilization protocol	Number of explants	Survival of explants (%)
10	37	95
11	37	62
12	15	93

The best result was achieved with the sterilization protocol number 10. 95 percent of the explants were successfully cultivated. This protocol was used for *in vitro* cultivation of the alpine plant samples collected from the Rax plateau.

Plant tissue culture from alpine plant samples from Rax plateau

The number of explants and the percentage of survival evaluated after 16 weeks were recorded (Table 7). Table 8 shows the number of explants on MS media supplemented with 5 µl 2,4-D for callus induction and the success of this induction. The number of cultivated explants per plant depended on the size and number of usable rosettes of the collected plants.

Table 7: Number of explants from *Leontopodium nivale* subsp. *alpinum* samples.

Plant sample ID	Number of explants (MS Ø)	Percentage of survival
LN1	17	41
LN2	4	25
LN3	8	50
LN4	14	14
LN5	5	60

Table 8: Number of explants from alpine plant samples on MS supplemented with 2,4-D

Plant sample ID	Number of explants (MS + 2,4-D)	Callus induction (yes/no)
LN1	27	no
LN2	18	yes
LN3	27	yes
LN4	19	no
LN5	26	yes

Figure 10: Plant tissue culture of *Leontopodium nivale* subsp. *alpinum* (plant sample 5)

Callus (Figure 11) was induced by auxin (2,4-D) and appeared at different times with an irregular growth, some explants showed growing callus after two weeks and other explants only after months. Callus induction from leaves of plant 1 and 4 failed, no callus cultures were observed. The leaf explants of all other plant samples (LN2, LN3 and LN5) produced calli.
Callus cultures were transferred onto fresh solid MS-2,4-D at monthly intervals.

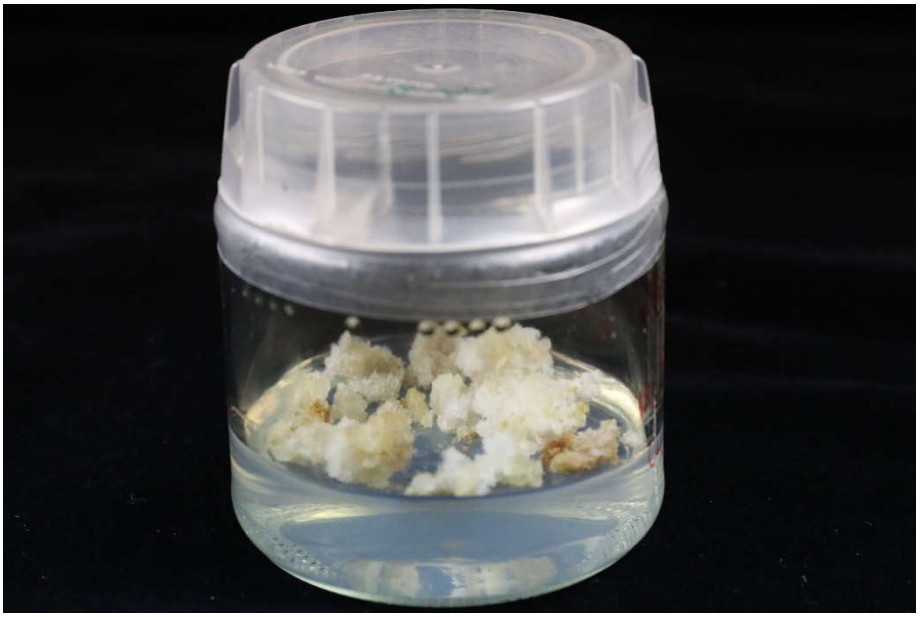

Figure 11: Callus culture of *Leontopodium nivale* subsp. *alpinum* (plant sample 2)

Maceration of plant tissue

Three different types of maceration tubes (Table 9) were tested for their suitability for different plant tissue types (leaves, rhizomes, roots) from Edelweiss. The bead maceration tubes (Precellys Bertin Technology, France) were selected because of their suitability for grinding all three different types of tissue.

Table 9: Bead maceration tubes (Precellys, city, country) tested for tissue maceration.

Type of Tubes	Prefilled bead
CK14	1,4 mm ceramic (zirconium oxide) beads
CK28-R[1]	2,8 mm ceramic beads
CKMix50-R[1]	2,8 mm and 5,0 mm ceramic beads

[1]R… reinforced

Non-sterilized plant tissues were cut into small pieces and approximately 120 mg were placed in 2 ml tubes containing 1 ml of 20% glycerol. For the fragmentation of the plant tissues the maceration was performed in a Precellys® 24 tissue homogenizer. The maceration was performed cumulative, meaning the maceration was done repeatedly with different agitation speeds and intervals (Table 10). The aim was to find the appropriate type of tubes for leaves, rhizomes and roots maceration.

Each macerated tissue was evaluated macroscopically and microscopically. Homogeneity of the macerates was evaluated by visual inspection; the homogeneity was important for pipetting of the macerate to be able to make serial dilutions. The microscopical criteria for macerates included cell clusters with less than three connecting cells and that the central cylinder of roots was disintegrated. These criteria ensured the efficient release of endophytic microorganisms from all tissues.

Table 10: Test for appropriate type of tube

Tube type	Plant tissue	Weight (mg)	Agitation speed (rpm)	Agitation interval (sec)
CK14	Leaves	164		
CK14	Rhizomes	216		
CK14	Roots	286	2500	20
CK28-R	Leaves	150	4000	20
CK28-R	Rhizomes	142	4000	60
CK28-R	Roots	165	6000	40
CKMix50-R	Leaves	186	6000	40
CKMix50-R	Rhizomes	96		
CKMix50-R	Roots	152		

Macerates were transferred onto Petri dishes (Figure 12). Macerates were subsequently diluted with 1 ml dH_2O and the suspension was mixed by gently shaking to be able to conduct the visual evaluation of the macerates.

Figure 12: Diluted macerates grinded with CKMix50-R tubes

The maceration with CK14 tubes showed in all three tissue types an initial grinding, but most plant cells remained intact, large cell clusters were visible.
Roots were appropriately macerated with CK28-R tubes, while the CKMiX50-R tubes resulted a lower disintegration of the central cylinder. The best results for leaves and rhizomes were obtained by using CKMix50-R tubes. However, the settings of the Precellys® 24 tissue homogenizer had to be optimized for better homogeneity and the defragmentation of cell clusters.
To evaluate if the high amount of plant material added to the maceration tubes in the previous experiment was the reason for poor maceration results, we repeated the maceration with a lower amount of plant material. The tubes were filled with only 90 mg of plant material and otherwise treated the same way as before (Table 11). The macerated tissues were again evaluated macroscopically and microscopically.

Table 11: Testing tube filling capacity and maceration

Tube type	Plant tissue	Weight (mg)	Agitation speed (rpm)	Agitation interval (sec)
CK14	Leaves	96	2500	20 sec
			4000	20 sec
CK28-R	Leaves	95	4000	60 sec
			6000	40 sec
CK50Mix-R	Leaves	93	6000	40 sec

The filling quantity of the maceration tubes had no effect on the outcome, the macerates showed a similar result as the test before, therefore it was important to optimize the maceration settings for better maceration results.

Experiments testing different maceration settings

In the next experiment, different settings of the Precellys®24 homogenizer were tested (Table 12). The aim was to find the optimal speed and duration for each plant tissue type. The optimal point between agitation duration and speed was reached when the macerate had a minimal temperature rise of the tube content and the macerates showed homogeneity. The maceration was performed step-wise. After every maceration cycle the macerate was evaluated for homogeneity microscopically.

Table 12: Settings of maceration

Tube type	Plant tissue	Weight (mg)	Agitation speed (rpm)	Agitation interval (sec)
CKMix50-R	Leaves	101	4000	40
			4500	20
			5000	20
CKMix50-R	Rhizomes	130	6000	40
			6500	40
			6800	30
CK28-R	Roots	101	6000	40
			6500	40
			6500	20

Table 13 shows the setting with the best maceration results for this experiment. Except for the rhizomes, the results for other tissue types were not fully satisfactory. The cell clusters of the leave macerate contained more than 3 cells. The central cylinders of the roots were not fully disintegrated with the setting of the second cycle (6500 rpm, 40 seconds), in addition the third cycle (6500 rpm, 20 seconds) showed no significant differences in maceration efficiency compared to the second cycle.

Table 13: Best maceration settings of the previous experiment

Plant tissue	Agitation speed (rpm)	Agitation interval (sec)
Leaves	5000	20
Rhizomes	6800	30
Roots	6500	40

Further optimization was done by the final testing to reach appropriate maceration results.

Final testing of combined methods and optimization of maceration settings

Finally, the combination of surface sterilization with the maceration was tested. The settings of the Precellys® 24 homogenizer have been optimized for the maceration run. Two different maceration settings were tested, to find the best non-cumulative maceration for each tissue. Plant samples were separated into aboveground and belowground tissues. After surface sterilization using the optimal protocol for aboveground and belowground tissues (surface sterilization protocol No. 10 and 29 respectively), they were prepared as described above using approximately 100 mg of plant material and 20 % glycerol for maceration. The tubes were stored in on ice before the maceration procedure.
The maceration was performed with the following settings (Table 14).

Table 14: Settings of final testing

Setting No.	Plant tissue	Tube type	Weight (mg)	Agitation speed (rpm)	Agitation interval (sec)
1	Leaves	CKMix50-R	112	5000	40
2	Leaves	CKMix50-R	150	6000	40
1	Rhizomes	CKMix50-R	77	6800	30
2	Rhizomes	CKMix50-R	63	6800	2x 30
1	Roots	CK28-R	94	6500	30
2	Roots	CK28-R	92	6800	30

100 µl of each macerate were added to 900 µl 20 % glycerol and dilutions up to 10^{-2} were made. 100 µl of each dilution were plated onto PDA and TSA as growth media for endophytes. The plates were incubated at approximately 10°C for avoiding fast bacterial and fungal overgrowth, to be able to count single colonies. After four days of incubation endophytic organisms of rhizomes had to be evaluated, otherwise bacterial overgrowth hampered the counting of the colonies. Endophytes of leaves and roots were evaluated after five days. Endophyte colonies were counted on each plate to evaluate the efficacy of the maceration process (Table 15). In this experiment the optimal grinding of plant tissues was examined, whereby the most endophytes could be released from the macerated plant tissues.

Table 15: Number of growing colonies on TSA and PDA appearing on TSA and PDA media after five days of cultivation evaluating method 1 and 2.

Tissue	Dilution	Colonies on TSA	Colonies on PDA
Leaves 1	10^{-0}	46	33
	10^{-1}	11	9
	10^{-2}	0	0
Leaves 2	10^{-0}	18	2
	10^{-1}	2	1
	10^{-2}	2	0
Rhizomes 1	10^{-0}	146	29
	10^{-1}	34	10
	10^{-2}	6	3
Rhizomes 2	10^{-0}	66	10
	10^{-1}	12	0
	10^{-2}	1	1
Roots 1	10^{-0}	32	0
	10^{-1}	24	0
	10^{-2}	23	0
Roots 2	10^{-0}	11	0
	10^{-1}	9	1
	10^{-2}	8	0

Table 16 shows counted numbers of morphologically different colonies of endophytes. Evaluated differences for endophytic bacteria were colony size,

color and shape. Endophytic fungi were evaluated based on morphological characteristics of reproductive structures and mycelia.

Table 16: Number of morphologically different colonies appearing on TSA and PDA media after five days of cultivation evaluating method 1 and 2.

Tissue	Dilution	Colonies on TSA	Colonies on PDA
Leaves 1	10^{-0}	7	4
	10^{-1}	4	3
	10^{-2}	0	0
Total number		11	7
Leaves 2	10^{-0}	3	1
	10^{-1}	2	1
	10^{-2}	2	0
Total number		7	2
Rhizomes 1	10^{-0}	6	2
	10^{-1}	7	6
	10^{-2}	5	4
Total number		18	12
Rhizomes 2	10^{-0}	6	4
	10^{-1}	4	6
	10^{-2}	4	3
Total number		14	13
Roots 1	10^{-0}	5	0
	10^{-1}	2	0
	10^{-2}	2	0
Total number		9	0
Roots 2	10^{-0}	4	0
	10^{-1}	1	1
	10^{-2}	2	0
Total number		7	1

A higher number of endophytic microorganisms could be observed with maceration setting No. 1 of each plant tissue (Table 15). Moreover, Table 16 shows that method 1 have resulted in a higher number of morphologically different endophytes.

The previous experiments showed that the following methods achieved the best results (Table 17) and were used for the experiments with the alpine plant samples from the mountain Rax.

Table 17: Surface sterilization and maceration protocol for the alpine plant samples

Plant tissue type	Surface sterilization agent	Time of exposure (min)	Maceration Tube	Rotational speed (rpm)	Duration (sec)
Leaves	0,1 % HgCl2	5	CKMix50-R	5000	40
Rhizomes	0,1 % HgCl2	10	CKMix50-R	6800	30
Roots	0,1 % HgCl2	10	CK28-R	6500	30

Isolates of Endophytes from alpine *L. nivale* subsp. *alpinum*

The establishment of an optimized protocol for the surface sterilization and maceration protocol of Edelweiss enabled the successful isolation and cultivation of 649 endophytic organisms on six different selective media: HV agar, GAC agar, ISP 2 agar, Kings B agar, PDA and SNA. Originally, a total of 833 isolates were picked from the isolation plates, but 174 of them could not be further cultivated or a pure culture could not be obtained due to contamination. From this point on, we will only refer to the 659 successfully cultivated endophytic isolates because in further studies just these isolates can be sequenced, and their phylogenetic diversity clarified. Furthermore 50 endophytic isolates were identified by Dr. Martina Oberhofer as fungal species and will not further discussed in this work.

Isolates allocated to plant samples and plant tissues

The number of isolated endophytes depended on the type of plant tissues. Figure 13 shows that most isolates originated from rhizomes. Only a small number of bacteria could be isolated from the roots the higher diluted macerate plates, because their growth was inhibited by antibiotic properties of the root macerate itself. One alga was isolated from leaves of plant sample LN 4.

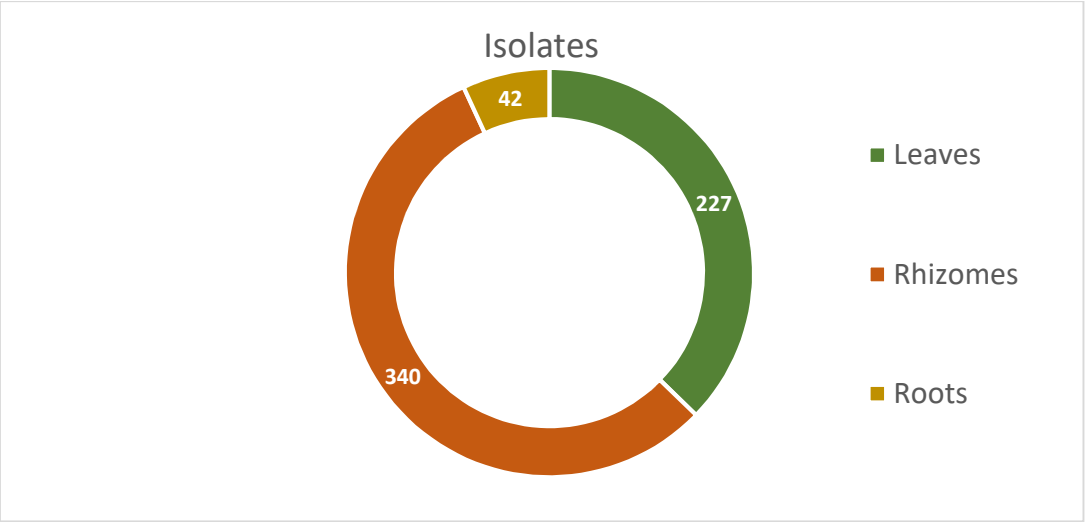

Figure 13: Total number of isolates allocated to plant tissue

Most endophytes, a total of 158, were isolated from the plant sample LN 5 (Figure 14).

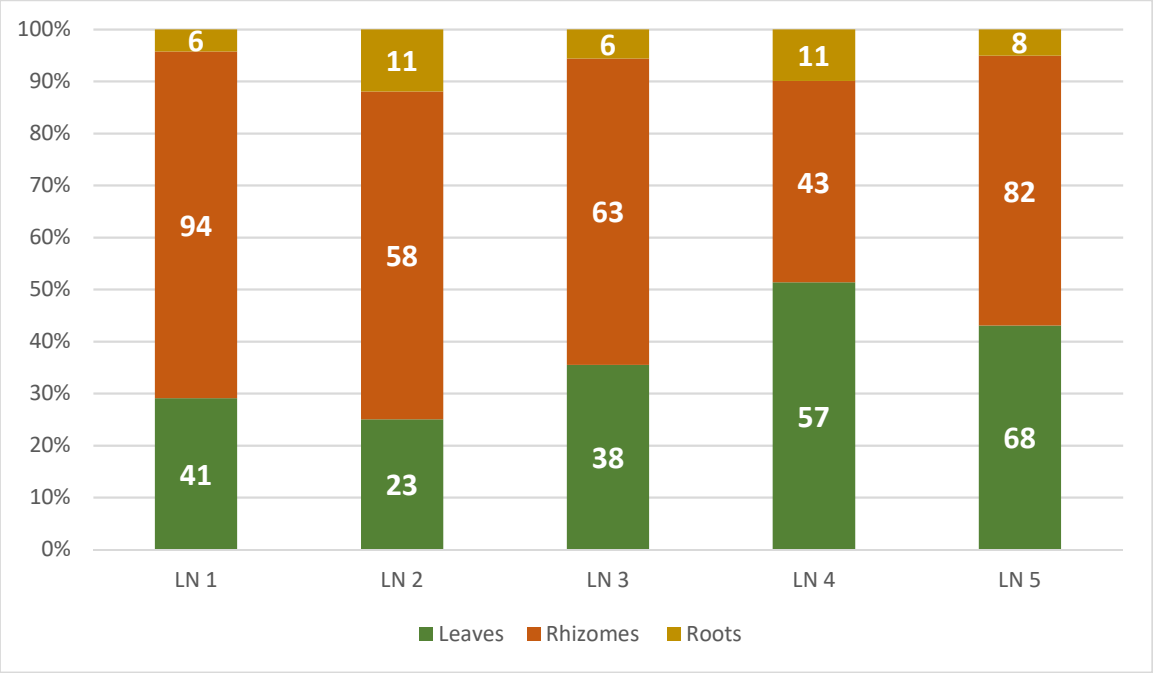

Figure 14: Number of isolates from different plant samples and tissues

Isolates allocated to selective media

Table 18 shows the number of isolates collected from six different selective media. The highest number of bacterial endophytes were isolated on media ISP2 and Kings B agar supplemented with Nystatin and Cycloheximide.

Table 18: Number of isolates on different selective media

Media	GAC NC	HV NC	ISP 2 NC	Kings B NC	PDA	SNA	Total
Number of Isolates	89	44	168	169	109	30	609

Endophytes identification

35 bacterial endophytes were successfully identified by 16S rRNA gene sequencing (Table 19). Consensus sequences were uploaded to the BLAST Databank of NCBI with the software Geneious Prime.

Table 19: Identification of isolates

Isolate ID	Plant ID	Tissue type	Identification	GenBank Acession No.
48	LN1	L	*Bacillus* sp.	MK954119
66	LN5	RH	*Pseudomonas* sp.	MH016575
78	LN4	RH	*Bacillus* sp.	MK388390
94	LN2	RH	*Pseudomonas* sp.	MK071278
97	LN4	RH	*Bacillus* sp.	MK480145
98	LN4	RH	*Viridibacillus* sp.	LK391524
100	LN4	RH	*Bacillus* sp.	JX402434
104	LN1	RH	*Bacillus* sp.	MH699234
114	LN1	RH	*Pseudomonas* sp.	JF778700
121	LN4	RH	*Pseudomonas* sp.	JQ977596
164	LN4	L	*Pseudomonas* sp.	KR922154
171	LN4	RH	*Bacillus* sp.	MH699234
174	LN3	RO	*Bacillus* sp.	MK954119
194	LN5	L	*Bacillus* sp.	MH687928
234	LN1	RO	*Braevibacterium* sp.	MF177857
278	LN4	RH	*Braevibacterium* sp.	MF177857
293	LN1	RH	*Luteibacter* sp.	CP017480
294	LN1	RH	*Pseudomonas* sp.	KR922149
298	LN2	L	*Pseudomonas* sp.	MH553941
306	LN2	RH	*Curtobacterium* sp.	KR906481
313	LN4	L	*Frigoribacterium* sp.	KR922060
446	LN4	L	*Luteibacter* sp.	CP017480
472	LN1	L	*Pseudomonas* sp.	NR_117820
475	LN4	L	*Pseudomonas* sp.	MH884040
477	LN4	L	*Pseudomonas* sp.	MK388374
523	LN1	L	*Duganella* sp.	NR_114106
654	LN2	RH	*Braevibacterium* sp.	MF537177
655b	LN2	RH	*Pseudomonas* sp.	MG576175
656	LN1	RH	*Luteibacter* sp.	MK559966
687	LN1	L	*Bacillus* sp-	MK954119
698	LN1	RH	*Terriglobus* sp.	NR_043918
718	LN3	L	*Pantoea* sp.	MH884045
753	LN4	RO	*Luteibacter* sp.	KY682049
767	LN4	RO	*Terriglobus* sp.	MG780331

Discussion

The need for novel bioactive compounds to treat diseases in humans, livestock and plants is ever growing (Alvin et al. 2014). Endophytic microorganisms from medicinal plants are a promising source for the production of bioactive secondary metabolites (Huang et al. 2007). The traditional medicinal plant *Leontopodium nivale* subsp. *alpinum* resides in the subalpine and alpine vegetation zones of the European mountain ranges. The native habitat of the plant is characterized by strongly fluctuating microclimatic conditions and a fragile ecosystem (Sun et al. 2014). Endophytic communities enhance fitness and the adaptation of plants in such high-stress environments (Truyens et al. 2015). Therefore, in the current project, we isolated endophytic microorganisms from *Leontopodium nivale* subsp. *alpinum* in order to study their potential for production of novel compounds in the future. To our knowledge, this study is the first to describe the isolation of endophytic microorganisms from *Leontopodium nivale* subsp. *alpinum*.

A total number of 833 endophytic microorganisms were isolated, and 659 of them were successfully further cultivated.

However, in this study apparently only a part of the possible range of endophytic microorganisms in Edelweiss could be isolated, since the seasonal endophytic communities in plants show a high variation. According to Martins et al. (2016), both the geographic location and the season of harvest influence the endophytic community, whereby the endophytic diversity decreases from spring to fall while the abundance of endophytes increases. An issue for further study will be a large-scale isolation of endophytes at different seasons over several years, to discover the entire cultivable endophytic diversity and to reveal, which endophytic species reside their whole life within the plant, and which are just temporary present.

Surface sterilization of the plant tissue was necessary to eliminate the epiphytes in order to be able to isolate only endophytic microorganisms. Environmental factors like microclimatic conditions and the season´s influence the endophytic communities inhabiting the plant and on the epiphyte concentration on the surface of the plant.

The evaluation of the sterilization process was first done by plating 0,5 cm^2 pieces of surface-sterilized plant tissues onto PDA and TSA media. This method was not

evaluated because a clear differentiation between contaminants and endophytic microorganisms emerging from the surface was not possible. Therefore, a combination of imprinting the sterilized plant tissue and plating out the washing water from the last washing step onto TSA and PSA were used.

According to Hallmann et al. (1997) the highest population density of endophytic microorganisms can be generally observed in the root system of the host plant and would decline from the stems to the leaves. Two different surface sterilization protocols were developed, one for the leaves and the second one for the belowground tissue of *Leontopodium nivale* subsp. *alpinum*. In this work, the lowest number of endophytes could be isolated from the roots. This may be an indication for a too stringent sterilization protocol used for the root material. In order to increase the yield of endophytic microorganisms from roots, it could be helpful to sterilize roots separately from the rhizomes with an optimized surface sterilization protocol. In further studies it would also useful to test whether secondary metabolites of the Edelweiss root tissue affect the number of isolated endophytic microorganisms.

In this study, six different selective media were used for the isolation of endophytes. Between 30 (SNA) and 169 (Kings B) endophytes could be isolated und successfully cultivated with these six media. The choice of selective media is an important criterion for the isolation process. In a further project, 16S-rRNA gene sequencing and a phylogenetic analysis of all isolates from this study will be conducted. These results will clarify the phylogenetic diversity of endophytic microorganisms to verify if the selected media are able to cover a broad range of different endophytes.

A second aim of this study was the establishment of a plant tissue culture and the callus induction for *Leontopodium nivale* subsp. *alpinum* from the Rax mountains. The *in vitro* cultivation as well es the callus induction could be successfully accomplished. The callus induction from the leaflets appeared at different times. The optimization of the callus induction protocol regarding the type of used phytohormones as well as their concentration could be required in future studies. An issue for further studies could also be to investigate to which extent the physiological state or the genetic background of the collected plants affects callus induction.

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Appendices

Appendix A

Extract from the “Artenschutzverordnung” of Lower Austria (Page 1, 2 and 5 of 18)

RIS

Landesrecht Niederösterreich

Gesamte Rechtsvorschrift für NÖ Artenschutzverordnung, Fassung vom 03.04.2019

Langtitel

NÖ Artenschutzverordnung
StF: LGBl. 5500/2-0
[CELEX-Nr.: 392L0043, 397L0062, 379L0409, 381L0854, 391L0244, 394L0024, 397L0049]

Präambel/Promulgationsklausel

Die NÖ Landesregierung hat am 5. Juli 2005 aufgrund des § 18 Abs. 2 und 3 des NÖ Naturschutzgesetzes 2000, LGBl. 5500–3, verordnet:

Text

§ 1

Gegenstand

Diese Verordnung dient dem Schutz **wildwachsender Pflanzen** und **freilebender Tiere**.

§ 2

Gänzlich geschützte Pflanzenarten

Gänzlich geschützt sind die in **Anlage 1** angeführten wildwachsenden Pflanzenarten.

§ 3

Gänzlich geschützte Tierarten

Gänzlich geschützt sind die in **Anlage 2** angeführten freilebenden Tierarten.

§ 4

Umgesetzte EG-Richtlinien

Durch diese Verordnung werden folgende Richtlinien der Europäischen Gemeinschaft umgesetzt:

1. Richtlinie 92/43/EWG des Rates vom 21. Mai 1992 zur Erhaltung der natürlichen Lebensräume sowie der wildlebenden Tiere und Pflanzen, ABl.Nr. L 206 vom 22. Juli 1992, S. 7;
2. Richtlinie 97/62/EG des Rates vom 27. Oktober 1997 zur Anpassung der Richtlinie 92/43/EWG zur Erhaltung der natürlichen Lebensräume sowie der wildlebenden Tiere und Pflanzen an den technischen und wissenschaftlichen Fortschritt, ABl.Nr. L 305 vom 8. November 1997, S. 42;
3. Richtlinie 79/409/EWG des Rates vom 2. April 1979 über die Erhaltung der wildlebenden Vogelarten, ABl.Nr. L 103 vom 25. April 1979, S. 1;
4. Richtlinie 81/854/EWG des Rates vom 19. Oktober 1981 zur Anpassung, aufgrund des Beitritts Griechenlands, der Richtlinie 79/409/EWG über die Erhaltung der wildlebenden Vogelarten, ABl.Nr. L 319 vom 7. November 1981, S. 3;
5. Richtlinie 91/244/EWG der Kommission vom 6. März 1991 zur Änderung der Richtlinie 79/409/EWG des Rates über die Erhaltung der wildlebenden Vogelarten, ABl.Nr. L 115 vom 8. Mai 1991, S. 41;
6. Richtlinie 94/24/EG des Rates vom 8. Juni 1994 zur Änderung von Anhang II der Richtlinie 79/409/EWG über die Erhaltung der wildlebenden Vogelarten, ABl.Nr. L 164 vom 30. Juni 1994, S. 9;
7. Richtlinie 97/49/EG der Kommission vom 29. Juli 1997 zur Änderung der Richtlinie 79/409/EWG des Rates über die Erhaltung der wildlebenden Vogelarten, ABl.Nr. L 223 vom 13. August 1997, S. 9.

§ 5

Schlussbestimmung

Mit dem Inkrafttreten dieser Verordnung tritt die Verordnung über den Schutz wildwachsender Pflanzen und freilebender Tiere, LGBl. 5500/2–2, außer Kraft.

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Anlage 1

Gänzlich geschützte Pflanzenarten

Legende:

Art	Die Pflanzenart wird mit dem deutschen und dem wissenschaftlichen Namen bezeichnet. Prioritäre Arten nach Anhang II lit.b der Fauna-Flora-Habitat-Richtlinie (§ 9 Abs. 2 Z 1 NÖ NSchG 2000) sind mit einem Sternchen (*) gekennzeichnet.
FFH	Pflanzenarten, die in den Anhängen II lit.b oder IV lit.b der Fauna-Flora-Habitat-Richtlinie angeführt sind, werden mit „X" bezeichnet.
Rote Liste	„Rote Listen" dokumentieren den Seltenheits- oder Bedrohungsgrad von Pflanzen- und Tierarten, basierend auf naturwissenschaftlichen Fachdaten. Diese Pflanzen sind wegen ihrer Seltenheit oder Bedrohung ihres Bestandes (§ 18 Abs. 2 Z 1 NÖ NSchG 2000) angeführt und betreffen grundsätzlich die Kategorien „0" = „ausgestorben oder verschollen" und „1" = „vom Aussterben bedroht".
pflückgefährdet	Pflanzenarten der „Roten Listen" und weitere Arten, die aufgrund des optischen Erscheinungsbildes und aufgrund von Traditionen einer Gefährdung durch übermäßige Entnahme unterliegen, werden mit „X" bezeichnet.

Art		**FFH**	**Rote Liste**	**pflück-gefährdet**
Gefäßpflanzen				
Duft-Becherglocke	Adenophora liliifolia		1	
Frühlings-Adonisröschen	Adonis vernalis			X
Kamm-Quecke	Agropyron pectinatum		1	
Kornrade	Agrostemma githago		1	
Zierlicher Nelkenhafer	Aira elegantissima		1	
Blasser Eibisch	Alcea biennis		1	
Gras-Froschlöffel	Alisma gramineum		1	
Sand-Steinkraut	Alyssum montanum subsp.gmelinii		1	
Acker-Mannsschild	Androsace maxima		1	
Steppen-Windröschen	Anemone sylvestris			X
Astlose Zaunlilie	Anthericum liliago		0	
Lückigen-Windhalm	Apera interrupta		1	
Kleinfrüchtiges Ohmkraut	Aphanes inexspectata		1	
Echte Sellerie	Apium graveolens		0	
Kriechende Sellerie	Apium repens	X	1	
Akelei, alle Arten	Aquilegia spp.			X
Gerards Gänsekresse	Arabis nemoralis (planisiliqua)		1	
Kampfer-Beifuß	Artemisia alba		0	
Schlitzblättriger Beifuß *	Artemisia laciniata	X	0	
Waldsteppen-Beifuß *	Artemisia pancicii	X		
Salz-Beifuß	Artemisia santonicum		1	
Acker-Meier	Asperula arvensis		0	
Graue Aster	Aster canus		1	
Ungarische Salzaster	Aster tripolium subsp.pannonicus		1	
Borsten-Tragant	Astragalus asper		1	
Dänischer Tragant	Astragalus danicus		1	
Boden-Tragant	Astragalus exscapus		1	
Furchen-Tragant	Astragalus sulcatus		1	
Ungarischer Blasen-Tragant	Astragalus vesicarius		1	
Rosen-Melde	Atriplex rosea		1	
Sand-Radmelde	Bassia (Kochia) laniflora (arenaria)		1	
Zwerg-Birke	Betula nana			X
Aufrechte Falzblume	Bombycilaena (Micropus) erecta		0	
Ästige Mondraute	Botrychium matricariifolium		1	
Vielspaltige Mondraute	Botrychium multifidum		1	
Acker-Trespe	Bromus arvensis		0	
Ungarische Trespe	Bromus pannonicus		1	

Art		FFH	Rote Liste	pflück-gefährdet
Sumpf-Platterbse	Lathyrus palustris		1	
Sumpfporst	Ledum palustre			X
Reisquecke	Leersia oryzoides		1	
Edelweis	Leontopodium alpinum			X
Durchwachsene Kresse	Lepidium perfoliatum		0	
Sommer-Knotenblume	Leucojum aestivum			X
Sibirischer Goldkolben	Ligularia sibirica	X		X
Feuer-Lilie	Lilium bulbiferum			X
Türkenbund-Lilie	Lilium martagon			X
Acker-Leinkraut	Linaria arvensis		1	
Liegendes Büchsenkraut	Lindernia procumbens		0	
Ausdauernder Lein	Linum perenne subsp.perenne		1	
Glanzstendel	Liparis loeselii	X	0	
Lein-Lolch	Lolium (temulentum) remotum		0	
Taumel-Lolch	Lolium (temulentum) temulentum		0	
Filz-Apfel	Malus dasyphylla		1	
Gewöhnlicher Andorn	Marrubium vulgare		1	
Hohes Perlgras	Melica altissima		1	
Hohldotter	Myagrum perfoliatum		1	
Deutsche Tamariske	Myricaria germanica		0	
Kleines Nixenkraut	Najas minor		1	
Gelbe Teichrose	Nuphar lutea			X
Kleine Teichrose	Nuphar pumila		0	X
Große Seerose	Nymphaea alba			X
Kleine Seerose	Nymphaea candida		0	X
Seekanne	Nymphoides peltata		1	
Röhrige Rebendolde	Oenanthe fistulosa		0	
Bocks-Hauhechel	Ononis arvensis (hircina)		1	
Sand-Lotwurz	Onosma arenarium subsp. arenarium		1	
Österreichische Lotwurz	Onosma arenarium subsp. austriacum		1	
Alle Orchideen	Orchidaceae		0 bis -	X
Wanzen-Knabenkraut	Orchis coriophora		1	
Sumpf-Knabenkraut	Orchis palustris (laxiflora ssp. pal.)		1	
Spitzels Knabenkraut	Orchis spitzelii		0	
Grüner Milchstern	Ornithogalum boucheanum		1	
Pyramiden Milchstern	Ornithogalum brevistylum		0	
Sand-Sommerwurz	Orobanche arenaria		1	
Weißwollige Sommerwurz	Orobanche caesia		1	
Bläuliche Sommerwurz	Orobanche coerulescens		1	
Beifuß-Sommerwurz	Orobanche loricata		0	
Bitterkraut-Sommerwurz	Orobanche picridis		1	
Hanf-Sommerwurz	Orobanche ramosa		0	
Echter Haarstrang	Peucedanum officinale		1	
Ungarischer Dünnschwanz	Pholiurus pannonicus		0	
Sand-Wegerich	Plantago (indica) arenaria		1	
Hochstieliger Wegerich	Plantago altissima		1	
Strauch-Wegerich	Plantago sempervivens		0	
Schmalblütiger Wegerich	Plantago teuiflora		0	
Bläuliches Rispengras	Poa (pratensis) subcoerulea		1	
Schlitzblättriger Stielsame	Podospermum laciniatum (Scorzonera lac.)		0	
Acker-Knorpelkraut	Polycnemum arvense		1	
Warzen-Knorpelkraut	Polycnemum verrucosum		1	
Ungarischer Knöterich	Polygonum bellardii		1	
Spitzblättriges Laichkraut	Potamogeton acutifolius		1	
Gefärbtes Laichkraut	Potamogeton coloratus		1	

Appendix B

AMT DER NIEDERÖSTERREICHISCHEN LANDESREGIERUNG
Gruppe Raumordnung, Umwelt und Verkehr
Abteilung Naturschutz
3109 St. Pölten, Landhausplatz 1

Amt der Niederösterreichischen Landesregierung, 3109

Universität Wien
Fakultät für Lebenswissenschaften
Ao. Univ.-Prof. Dr. Michael Kiehn
Rennweg 14
1030 Wien

Kennzeichen (bei Antwort bitte angeben)	Beilagen	Bezug	Bearbeiter/in	(0 27 42) 9005 Durchwahl	Datum
RU5-BE-182/010-2015	2 + ZS		Karin Altinger-Probst	15215	24. Februar 2015

E-Mail: post.ru5@noel.gv.at - Telefax 02742/9005/15220
Internet: http://www.noe.gv.at DVR: 0059986
Bürgerservice-Telefon 02742/9005-9005

Betrifft
Universität Wien, Fakultät für Lebenswissenschaften, 1030 Wien, Ansuchen um Ausnahmegenehmigung gemäß § 20 Abs. 4 i.V.m. § 11 Abs. 6 NÖ NSchG 2000 und §§ 5 und 6 NÖ Nationalparkgesetz; Bescheid

Bescheid

Über Ihren Antrag vom 16. Jänner 2015 um Erteilung einer Ausnahmegenehmigung vom Eingriffsverbot sowie Antrag um Sammelbewilligung für Mitarbeiter des Botanischen Gartens für wissenschaftliche Untersuchungen im Bundesland Niederösterreich, einschließlich Naturschutzgebiete und Nationalparks für den Zeitraum von 2015 bis 2019 wird wie folgt entschieden:

Spruch

Den Mitarbeitern des Botanischen Gartens der Universität Wien wird die Ausnahmegenehmigung vom Eingriffsverbot sowie die Sammelbewilligung für Samen und Pflanzen für wissenschaftliche Untersuchungen im Bundesland Niederösterreich, einschließlich Nationalparks und Naturschutzgebiete, ausgenommen Wildnisgebiet Dürrenstein, für den Zeitraum von 2015 bis 2019 erteilt, wobei folgende Auflagen einzuhalten sind:

1. Pflanzen, Steckling und Samen dürfen nur entnommen werden, wenn absehbar ist, dass eine Schädigung der Population und des Standortes ausgeschlossen werden kann.

2. Für die Sammeljahre 2015 – 2019 ist einmal jährlich ein Sammelbericht vorzulegen, dessen Inhalte sich an dem aus dem Jahre 2014 zu orientieren haben: Sammelnum-

mer, Familie, Gattung, Art, Sammelort (genaue Lage), NUTS-Code, Sammler, Sammeldatum.

3. Vor Exkursionen zur Entnahme von Samen, Stecklingen und Pflanzen der jeweiligen Arten sind die jeweiligen Nationalparkverwaltungen zu informieren und ist dabei um Erlaubnis zu bitten.

Hinweis:
Die 10-Jahres-Managementpläne der Nationalparks regeln auch die Betretungs- und Sammelmöglichkeit im Nationalpark. Deshalb sind bei der Sammlung von Pflanzen oder Tieren die Bestimmungen des jeweils gültigen 10-Jahres-Managementplanes zu beachten.

Die Bewilligung gilt für folgende Personen unter der Adresse Botanischer Garten, Universität Wien, Rennweg 14, 1030 Wien:

Dipl.-Ing. Frank SCHUMACHER
Franz TOD
Robert WERNERT
Ao. Univ.-Prof. Dr. Michael KIEHN
Gast-Doz. Prof. Dr. Arndt KÄSTNER
Isabella SIRNY
David PREHSLER
Mag. Margarita LACHMAYER
Dipl.-Ing. Barbara KNICKMANN
Michael MÜNCH

Sie sind verpflichtet, folgende Verfahrenskosten für diese Bewilligung innerhalb von vier Wochen ab Zustellung dieses Bescheides zu bezahlen:

Landesverwaltungsabgabe € 109,--

Der Betrag von € 109,-- ist mit beiliegendem Zahlschein zu entrichten.

Rechtsgrundlagen:
für die Sachentscheidung:
§ 20 Abs. 4 i.V.m. § 11 Abs. 6 NÖ Naturschutzgesetz 2000, NÖ NSchG 2000, LGBl. 5500
§ 5 Abs. 4 und § 6 Abs. 4 NÖ Nationalparkgesetz, LGBl. Nr. 5505
für die Kostenentscheidung:
§ 1 des Landes- und Gemeinde-Verwaltungsabgabengesetzes, LGBl. 3800, i.V.m. § 1, Tarifpost 83 der NÖ Landes-Verwaltungsabgabenverordnung 2001, LGBl. 3800/1.

Begründung

Mit Schreiben vom 16. Jänner 2015 hat Herr Ao. Univ.-Prof. Dr. Michael Kiehn als Leiter des Botanischen Gartens der Universität Wien einen Antrag um Erteilung einer Ausnah-

megenehmigung vom Eingriffsverbot sowie einen Antrag um Sammelbewilligung für Samen und Pflanzen für Mitarbeiter des Botanischen Gartens für wissenschaftliche Untersuchungen im Bundesland Niederösterreich, einschließlich Naturschutzgebiete und Nationalparks für den Zeitraum von 2015 bis 2019 gestellt.

Zunächst ist festzuhalten, dass die Landesregierung gemäß § 20 Abs. 4 des NÖ Naturschutzgesetzes 2000 Ausnahmen von den Vorschriften nach § 18 gestatten kann, sofern es keine anderweitige zufrieden stellende Lösung gibt und unter der Bedingung, dass die Populationen der betroffenen Art in ihrem natürlichen Verbreitungsgebiet trotz der Ausnahmegenehmigung ohne Beeinträchtigung in einem günstigen Erhaltungszustand verweilen können. In der Bewilligung ist zumindest festzulegen,

1. für welche Arten die Ausnahme gilt,
2. die zugelassenen Fang- oder Tötungsmittel, -einrichtungen und -methoden und
3. welche Kontrollen vorzunehmen sind.

Weiters wird festgehalten, dass in Naturschutzgebieten jeder Eingriff in das Pflanzenkleid oder Tierleben und jede Änderung bestehender Boden- oder Felsbildungen verboten ist. Weiters ist das Betreten außerhalb der gemäß § 11 Abs. 5 NÖ NSchG 2000 in der Verordnung bezeichneten Wege und Bereiche verboten.

Gemäß § 11 Abs. 6 NÖ NSchG 2000 sind von der Landesregierung durch Bescheid Ausnahmen vom Eingriffsverbot nach Abs. 4, insbesondere für Zwecke der wissenschaftlichen Forschung zuzulassen, soweit dies mit dem Ziel der Schutzmaßnahme nicht im Widerspruch steht oder nachteilige Auswirkungen auf das Naturschutzgebiet durch Vorschreibung von Vorkehrungen weitgehend ausgeschlossen werden können.

Die Regelungen zur Naturzone in § 5 NÖ Nationalparkgesetz sehen vor, dass hier prinzipiell jeder Eingriff in die Natur und in den Naturhaushalt sowie jede Beeinträchtigung des Landschaftsbildes verboten ist. Ex lege Ausnahmen von diesem Verbot bestehen unter anderem für Organe der Nationalparkverwaltung zur Erfüllung der ihnen gesetzlich übertragenen Aufgaben sowie für Besucher zum Begehen der für sie bestimmten Wege. Weitere Ausnahmen sind zuzulassen, soweit dies mit den Zielen des Nationalparks nicht im Widerspruch steht oder nachteilige Auswirkungen auf den Nationalpark durch Vorschreibung von Vorkehrungen weitgehend ausgeschlossen werden können.

In der Naturzone mit Managementmaßnahmen - § 6 NÖ Nationalparkgesetz - bestehen zusätzlich ex lege Ausnahmen von dem in dieser Zone ebenfalls festgelegten grundsätzlichen Eingriffsverbot, auch hier sind weitere Ausnahmen insbesondere für Zwecke der wissenschaftlichen Forschung bescheidmäßig zuzulassen.

Die Behörde holte im Rahmen des Ermittlungsverfahrens ein Gutachten eines Amtssachverständigen für Naturschutz ein. Dieses Gutachten vom 26. Jänner 2015 lautet wie folgt:

„Mit Schreiben vom 16.01.2015, per Poststempel eingegangen am 21.01.2015, ersuchte Herr Ao. Univ.-Prof. Dr. Michael KIEHN als Leiter des Botanischen Gartens der Universität Wien und Herr Dipl.-Ing. Frank SCHUMACHER als stellvertretender Leiter der genannten

Institution um Ausstellung einer Sammelbewilligung für das Sammeln von Samen und Pflanzen aus wissenschaftlichen Gründen nach dem Naturschutz- und dem Nationalparkgesetz, für die nachstehend genannten Mitarbeiter des Botanischen Gartens für den Zeitraum von 2015 bis 2019.

Die Entnahme der Samen und Pflanzen geschieht nur, wenn absehbar ist, dass eine Schädigung der Population ausgeschlossen werden kann.

Gleichzeitig wurde ein Sammelbericht für das Jahr 2009 - 2014 abgegeben. Dieser ist bescheidgemäß durchgeführt und durch seine Genauigkeit entsprechend aussagekräftig.

Mit Schreiben vom 21. Jänner 2015 ersuchte die Naturschutzabteilung (Abt. RU5), Gruppe Raumordnung, Umwelt und Verkehr, um gutachtliche Stellungnahme zum obigen Betreff.

Insbesondere wird um Stellungnahme zu folgenden Beweisthemen ersucht:
1. zum Antrag auf Sammelbewilligung gemäß § 20 Abs. 4 NÖ NSchG 2000,
2. zur Ausnahmegenehmigung vom Eingriffsverbot in die Naturschutzgebiete gemäß § 11 Abs. 6 NÖ NSchG 2000,
3. zur Ausnahmegenehmigung vom Eingriffsverbot in Nationalparks gemäß §§ 5 und 6 NÖ Nationalparkgesetz.

Gemäß § 20 Abs. 4 NÖ Naturschutzgesetz kann die Landesregierung Ausnahmen von den Vorschriften nach § 18 gestatten, sofern es keine anderweitige zufrieden stellende Lösung gibt und unter der Bedingung, dass die Populationen der betroffenen Art in ihrem natürlichen Verbreitungsgebiet trotz der Ausnahmegenehmigung ohne Beeinträchtigung in einem günstigen Zustand verweilt.

Laut Ansuchen des Instituts für Botanik sollen nur **einzelne Exemplare** einiger pannonisch, collin und alpin verbreiteter Arten zur Auffrischung des vorhandenen Genpools im Botanischen Garten und zur behutsamen Erweiterung des Selbigen entnommen werden.

Im Speziellen wird die Pannonische Gruppe des HBVs ständig aktualisiert und ergänzt. Dazu ist die Anzucht der entsprechenden Pflanzen nötig.
Die Anzucht über Saatgut wird dabei grundsätzlich einer Entnahme von Pflanzen vorgezogen.

Diese Aktivitäten stehen im Zusammenhang mit bereits angelegten und geplanten Erhaltungsmaßnahmen von gefährdeten Arten Österreichs im Rahmen von ex-situ-Programmen, wie sie in der Konvention zur Biologischen Diversität (CBD) vorgesehen sind. Der Botanische Garten ist Partner von ENSCONET (http://www.ensconet.com/).

Vorgesehene Mitarbeiter:
Sammelaktivität aller Personen unter der Adresse: Botanischer Garten; Universität Wien, Rennweg 14, 1030 Wien:
Dipl.-Ing. Frank Schumacher
Franz Tod
Robert Wernert
Ao. Univ.-Prof. Dr. Michael Kiehn
Gast-Doz. Prof. Dr. Arndt Kästner

Isabella Sirny
David Prehsler
Mag. Margarita Lachmayer
Dipl.-Ing. Barbara Knickmann
Michael Münch

Gutachtliche Stellungnahme
a.) Sammelbewilligung gemäß § 20 Abs. 4 NÖ NSchG 2000
Da es sich bei den aufgelisteten Mitarbeitern um ausgewiesene Experten handelt, nur einzelne Exemplare entnommen werden, die Methode der Anzucht über Saatgut der Entnahme ganzer Pflanzen vorgezogen werden soll, und die Aktivitäten im Zusammenhang mit Programmen von Erhaltungssammlungen, wie sie in der Konvention zur Biologischen Diversität (CBD) vorgesehen sind, kann aus fachlicher Sicht davon ausgegangen werden, **dass wildlebende Pflanzenbestände durch die Sammelaktivitäten im beantragten Ausmaß für wissenschaftliche Zwecke nicht gefährdet werden.** Es kann deshalb eine Ausnahmegenehmigung erlassen werden.

b.) Ausnahmegenehmigung vom Eingriffsverbot in die Naturschutzgebiete gemäß § 11 Abs. 6 NÖ NSchG 2000
Die Aktivitäten der Antragsteller stehen nicht mit dem Ziel der Schutzmaßnahme im Widerspruch. Ebenso können nachteilige Auswirkungen auf die jeweiligen Naturschutzgebiete ausgeschlossen werden.

c.) Ausnahmegenehmigung vom Eingriffsverbot in Nationalparks gemäß §§ 5 und 6 NÖ Nationalparkgesetz
Die Aktivitäten der Antragsteller stehen nicht mit den Bestimmungen des NÖ Nationalparkgesetzes im Widerspruch. Nachteilige Auswirkungen auf die jeweiligen Nationalparks können ausgeschlossen werden.

Auflagen
1. Pflanzen, Steckling und Samen dürfen nur entnommen werden, wenn absehbar ist, dass eine Schädigung der Population und des Standortes ausgeschlossen werden kann.
2. Für das Sammeljahr 2015 - 2019 ist einmal jährlich ein Sammelbericht vorzulegen, dessen Inhalte sich an dem aus dem Jahre 2014 zu orientieren haben: Sammelnummer, Familie, Gattung, Art, Sammelort (genaue Lage), NUTS-Code, Sammler, Sammeldatum.
3. Vor Exkursionen zur Entnahme von Samen, Stecklingen und Pflanzen der jeweiligen Arten sind die jeweiligen Nationalparkverwaltungen zu informieren und ist dabei um Erlaubnis zu bitten."

Mit Schreiben vom 28. Jänner 2015 wurde dieses Gutachten im Rahmen des Parteiengehörs sämtlichen Parteien des Verfahrens zur Kenntnis gebracht und Gelegenheit zur Stellungnahme gegeben.

Die Nationalpark Donau-Auen GmbH nahm mit Schreiben vom 29. Jänner 2015 das Gutachten des Amtssachverständigen für Naturschutz zustimmend zur Kenntnis.

Aufgrund des festgestellten Sachverhaltes und des eingeholten Gutachtens des Amtssachverständigen für Naturschutz konnte den Mitarbeitern des Botanischen Gartens der Universität Wien die Ausnahmegenehmigung vom Eingriffsverbot zum Sammeln von Samen und Pflanzen für wissenschaftliche Untersuchungen im Bundesland Niederösterreich für den Zeitraum von 2015 bis 2019 erteilt werden.

Rechtsmittelbelehrung

Sie haben das Recht gegen diesen Bescheid **Beschwerde** zu erheben.

Die Beschwerde ist innerhalb von **vier Wochen** nach Zustellung dieses Bescheides **schriftlich oder in jeder anderen technisch möglichen Weise beim Amt der NÖ Landesregierung, Abteilung Naturschutz, einzubringen.**
Sie hat den Bescheid, gegen den sie sich richtet, und die Behörde, die den Bescheid erlassen hat, zu bezeichnen. Weiters hat die Beschwerde die Gründe, auf die sich die Behauptung der Rechtswidrigkeit stützt, das Begehren und die Angaben, die erforderlich sind, um zu beurteilen, ob die Beschwerde rechtzeitig eingebracht ist, zu enthalten.

Die Höhe der Pauschalgebühr für Beschwerden, Wiedereinsetzungsanträge und Wiederaufnahmeanträge (samt Beilagen) beträgt 30,-- Euro.

Hinweise:
Die Gebühr ist auf das Konto des Finanzamtes für Gebühren, Verkehrssteuern und Glücksspiel (IBAN: AT83 0100 0000 0550 4109, BIC: BUNDATWW) zu entrichten.
Als Verwendungszweck ist das Beschwerdeverfahren (Geschäftszahl des Bescheides) anzugeben.

Der Eingabe ist - als Nachweis der Entrichtung der Gebühr - der Zahlungsbeleg oder ein Ausdruck über die erfolgte Erteilung einer Zahlungsanweisung anzuschließen. Für jede gebührenpflichtige Eingabe ist vom Beschwerdeführer (Antragsteller) ein gesonderter Beleg vorzulegen.

Ergeht an:
1. NÖ Umweltanwaltschaft, Wiener Straße 54, 3109 St. Pölten
2. Nationalpark Donau-Auen GmbH, Schloss Orth, 2304 Orth an der Donau
3. Nationalpark Thayatal GmbH Nationalparkhaus, 2082 Hardegg
4. Schutzgebietsverwaltung Wildnisgebiet Dürrenstein GF Dipl.-Ing. Dr. Christoph Leditznig, Brandstatt 61, 3270 Scheibbs
   zur Kenntnis.
5. BD2 Sekretariat Naturschutz, z.H. Herrn Dr. Manfred Pöckl
   zur Kenntnis zu BD2-N-201/008-2005.

NÖ Landesregierung
Im Auftrag
Mag. H i e s b e r g e r

NIEDERÖSTERREICH
AMTSSIGNATUR

Dieses Schriftstück wurde amtssigniert.
Hinweise finden Sie unter:
www.noe.gv.at/amtssignatur

Appendix C

Isolation template: Endophytic microorganisms from respective tissue, plant and isolation media.

ID	Endophyte	Tissue	Plant LN	Dilutions	Media	Isolation	Comment	Equals
1	B	L	4	0	PDA	-		
2	B	L	5	-1	PDA	-		
3	B	L	1	0	PDA	-		
4	B	L	1	-2	PDA	-		20
5	B	L	1	-1	King B NC	-	Pseudomonas fluorescens	
6	B	L	1	0	King B NC	-	Pseudomonas fluorescens	
7	B	L	4	0	King B NC	+		
8	B	L	3	-1	King B NC	+		
9	B	L	3	-1	King B NC	+		
10	B	L	2	0	King B NC	+		
11	B	L	3	0	King B NC	+		
12	B	L	3	0	PDA	+		
13	B	L	1	0	ISP2 NC	+		
14	B	L	4	-1	ISP2 NC	+		17
15	B	L	3	0	ISP2 NC	+		19
16	B	L	4	0	ISP2 NC	+		
17	B	L	4	0	ISP2 NC	+	redundant	14
18	B	L	5	-2	ISP2 NC	+		51,195
19	B	L	3	-1	ISP2 NC	+	redundant	15
20	B	L	1	-1	PDA	-	redundant, fungal contamination, lost	4
21	B	L	3	-1	PDA	+		
22	B	L	5	-3	PDA	+		25
23	B	L	4	-2	PDA	-		
24	B	L	4	-1	PDA	-	Pseudomonas fluorescens	
25	B	L	5	-2	PDA	+	redundant	22
26	A	L	5	-2	PDA	+		
27	B	L	5	0	PDA	-	fungal contamination, lost	
28	B	L	5	0	PDA	+		
29	B	L	5	0	PDA	-	Pseudomonas fluorescens	
30	B	L	5	-2	GAC NC	+	nicht kategorisiert	
31	B	L	5	0	King B NC	-	Pseudomonas fluorescens	
32	B	L	5	0	King B NC	-	Pseudomonas fluorescens	
33	B	L	5	0	King B NC	+		
34	B	L	5	0	King B NC	-	Pseudomonas fluorescens	
35	B	L	5	-2	King B NC	-		
36	B	L	5	-3	King B NC	+		
37	B	L	2	-1	King B NC	-	Pseudomonas fluorescens	
38	B	L	2	-1	King B NC	-	Pseudomonas fluorescens	
39	B	L	2	-1	King B NC	+		
40	B	L	1	-2	King B NC	-	Pseudomonas fluorescens	
41	B	L	5	-1	King B NC	+		

84	B	RO	2	-1	King B NC	+		
85	B	RH	3	0	ISP2 NC	-		
86	B	RH	3	0	ISP2 NC	+		88, 92, 191
87	B	RH	5	-1	ISP2 NC	+		
88	B	RH	5	-1	ISP2 NC	+	redundant	86, 92, 191
89	B	RH	5	0	ISP2 NC	+		
90	B	RH	5	0	ISP2 NC	+		
91	B	RH	3	-1	ISP2 NC	-		
92	B	RH	3	-1	ISP2 NC	+	redundant	88, 86, 191
93	B	RH	2	-1	ISP2 NC	+		176
94	B	RH	2	-1	ISP2 NC	+		
95	B	RH	4	-3	ISP2 NC	+		100, 177
96	B	RH	4	-1	ISP2 NC	+		
97	B	RH	4	-1	ISP2 NC	+		
98	B	RH	4	-1	ISP2 NC	+		
99	B	RH	4	0	ISP2 NC	+		
100	B	RH	4	0	ISP2 NC	+	redundant	95, 177
101	B	RH	2	0	ISP2 NC	+		
102	B	RH	1	-2	ISP2 NC	+		105
103	B	RH	1	-2	ISP2 NC	+		
104	B	RH	1	-2	ISP2 NC	+		
105	B	RH	1	0	ISP2 NC	+	redundant	102
106	B	RH	1	0	ISP2 NC	+		
107	B	L	5	-2	GAC NC	+		
108	B	L	2	0	PDA	+		
109	B	L	2	-1	PDA	-		328
110	A	L	4	-2	PDA	+		
111a	B	RH	1	-1	PDA	+		
111b	F	RH	1	-1	PDA			
112	B	RH	1	-1	PDA	+		
113	B	RH	1	-1	PDA	+		
114	B	RH	1	-1	PDA	+		
115	A	L	0	0	PDA	-		204
116	B	L	1	-1	PDA	+		
117	A	L	1	-1	PDA	+		
118	B	RH	1	0	PDA	-		
119	B	RH	1	0	PDA	+		
120	F	RH	1	0	PDA			
121	B	RH	4	0	PDA	+		
122	B	RH	4	0	PDA	-	no growth on isolation media	
123	B	RH	4	-1	PDA	+		
124	B	RH	4	-1	PDA	-		

125	B	RH	4	-1	PDA	-	no growth on isolation media	
126	B	RH	3	0	PDA	+		
127	B	RH	4	-2	PDA	+		
128	B	RH	5	0	PDA	+	in freezer as no. 138	
129	B	RH	5	0	PDA	+	in freezer as no. 139	
130	B	RH	1	0	PDA	+		
140	B	L	4	-1	GAC NC	+	redundant	59
141	B	L	5	0	GAC NC	+		
142	A	L	5	0	GAC NC	+		
143	B	L	4	0	GAC NC	+		
144	B	L	4	0	GAC NC	+		418
145	B	L	1	0	GAC NC	+		
146	B	L	4	-2	GAC NC	+		
147	B	RH	3	-2	GAC NC	+		
148	B	RH	5	-1	GAC NC	+		
149	B	L	5	-1	GAC NC	+		
150	A	L	5	-1	GAC NC	+		
151	B	L	1	-1	GAC NC	+		
152	A	L	1	-1	GAC NC	+		
153	B	L	1	-2	GAC NC	+		424
154	B	RH	1	-3	King B NC	+		
155	B	RH	1	-3	King B NC	+		
156	B	RH	1	-3	King B NC	+		
157	B	L	1	0	King B NC	+		
158	B	RO	1	-2	King B NC	+		
159	B	RH	5	-3	King B NC	+	causes biocristallisation on KB agar	
160	B	RH	5	-2	King B NC	+		
161	A	RH	5	-2	King B NC	+		
162	B	L	3	-3	King B NC	+	causes biocristallisation on KB agar	
163	B	L	2	-2	King B NC	+		
164	B	L	4	0	King B NC	+		
165	B	L	1	-1	King B NC	+		
166	B	RH	4	-2	King B NC	+	crystals formed in antibiot. Interaction with 364	
167	B	RH	2	-1	King B NC	+		
168a	B	L	5	0	King B NC	-	no growth without 168b, lost	
168b	B	L	5	0	King B NC	+		
169	B	L	4	-1	King B NC	+		
170	B	RH	3	-2	King B NC	+		
171	B	RH	4	0	King B NC	+		
172	B	RH	4	-1	King B NC	+		
173	B	RH	3	-1	King B NC	+		
174	B	RO	1	-1	ISP2 NC	+		

175	B	L	1	0	ISP2 NC	+		
176	B	RO	2	-1	ISP2 NC	+	redundant	93
177	B	RH	4	-2	ISP2 NC	+	redundant	100, 95
178	B	RH	5	-2	ISP2 NC	+		
179	A	L	5	0	ISP2 NC	-		
180	B	L	5	-1	ISP2 NC	+		
181	B	L	3	0	ISP2 NC	+		
182	A	L	5	-2	ISP2 NC	+		
183	F	L	5	-3	SNA			
184	B	RH	3	0	ISP2 NC	+		
185	A	L	1	-1	ISP2 NC	+	no growth on isolation media	
186	B	L	1	-1	ISP2 NC	+	redundant	48, 49
187	B	RH	1	-1	ISP2 NC	+		
188	B	RH	1	-1	ISP2 NC	+		
189	B	RH	2	0	ISP2 NC	+		
190	B	L	4	-1	ISP2 NC	+		
191	B	RH	5	-2	ISP2 NC	+	redundant	92, 88
192	A	L	4	0	ISP2 NC	+		
193	B	L	4	0	ISP2 NC	+		
194	B	L	5	-2	ISP2 NC	+		
195	B	L	5	-2	ISP2 NC	+		18, 51
196	B	L	2	0	ISP2 NC	+		
197	B	L	2	0	ISP2 NC	+		
198	F	RH	5	-2	PDA			
199	B	RH	5	-2	PDA	+		209
200	B	RH	5	-2	PDA	-		201, 330
201	B	RH	5	-2	PDA	+	redundant	200, 330
202	F	RH	5	-1	PDA			
203	B	RH	5	-1	PDA	-		
204	A	L	4	-1	PDA	+	redundant	115
205	B	L	4	-1	PDA	+		
206	B	L	3	-1	PDA	+		211, 218
207	A	L	5	0	PDA	+		
208	B	RH	4	-1	PDA	+		
209	B	L	5	-1	PDA	+	redundant	199
210	B	L	3	0	PDA	-		
211	B	L	3	0	PDA	+	redundant	206, 218
212	A	L	1	0	PDA	-		
213	B	RH	5	0	PDA	-		
214	F	RH	5	0	PDA			
215	B	RH	4	-2	PDA	+		
216	B	L	4	0	PDA	-		

217	B	L	4	0	PDA	+		
218	B	L	3	-2	PDA	+		206, 211
219	A	L	5	-2	GAC NC	+		
220	B	L	4	0	GAC NC	+		
221	B	RH	2	-2	GAC NC	+		
222	B	RH	5	-2	GAC NC	-	Pseudomonas fluorescens	
223	A	L	4	-2	King B NC	+		
224	B	L	3	-2	King B NC	+		269, 283, 370, 378
225	B	RH	5	-1	King B NC	+		
226	B	RH	5	-1	King B NC	+		
227	B	L	2	0	King B NC	-	Pseudomonas fluorescens	
228	B	L	2	0	King B NC	-	Pseudomonas fluorescens	
229	B	RH	5	-2	King B NC	+		
230	B	RH	2	-2	King B NC	+		
231	B	RH	2	-2	King B NC	+		
232	B	L	2	-2	King B NC	+		
233	A	RH	1	-3	King B NC	+		
234	B	RO	1	-2	King B NC	+		
235	B	RH	2	-1	King B NC	+		
236	B	L	2	-2	PDA	+		241, 242
237	B	RH	2	-1	PDA	+		249
238	B	RH	3	-3	PDA	-		
239	B	RH	3	-3	PDA	+		254
240	B	L	4	-2	PDA	+		257, 260
241	B	L	2	-1	PDA	-	redundant	236, 242
242	B	L	2	0	PDA	-	redundant	236, 241
243	A	RH	1	-2	PDA	+		
244	B	RH	1	-2	PDA	+		
245a	B	RH	1	-2	PDA	-	Pseudomonas fluorescens	
245b	A	RH	1	-2	PDA	+		
246	B	RH	3	-2	PDA	+		252
247	B	RH	5	-3	PDA	+		
248	B	RH	5	-3	PDA	+		
249	B	RH	2	-2	PDA	+	redundant	237
250	B	L	5	-2	PDA	-		414
251	A	RH	3	-1	PDA	+		
252	B	RH	3	-1	PDA	+	redundant	246
253	B	RH	3	-1	PDA	+		
254	B	RH	3	0	PDA	+	redundant	239
255	F	RH	3	0	PDA			
256	B	L	3	-1	PDA	-		259
257	B	L	4	-1	PDA	-	redundant	24, 260

258	B	L	5	-1	PDA	-		
259	B	L	3	0	PDA	-	redundant	256
260	B	L	4	0	PDA	-	redundant	24, 257
261	B	RH	5	0	PDA	+		
262	B	RH	5	-1	PDA	+		
263	B	RH	5	-2	PDA	+		
264	B	RH	2	0	PDA	+		
265	B	RH	2	0	PDA	-		
266	B	RH	2	0	PDA	+		
267	B	RH	1	-3	PDA	+		
268	B	RH	2	-3	King B NC	+		
269	B	L	2	-1	King B NC	+		
270	B	L	1	-2	King B NC	+		
271	B	L	5	-1	King B NC	+	Pseudomonas fluorescens	
272	B	L	5	-1	King B NC	+	Pseudomonas fluorescens - REFERENZSTAMM	
273	A	L	1	-1	King B NC	+		
274	B	L	3	-1	King B NC	+		
275	B	RH	5	0	King B NC	+		
276	F	RH	5	0	King B NC			
277	B	RH	5	0	King B NC	+		
278	B	RH	4	0	King B NC	+		
279	B	L	4	-1	King B NC	+		
280	B	L	4	-1	King B NC	-	Pseudomonas fluorescens	
281	B	RH	2	-1	King B NC	+		
282	B	RH	2	-1	King B NC	-		
283	B	L	4	-2	King B NC	-		224, 269, 370, 378
284	B	L	2	-3	King B NC	-	Pseudomonas fluorescens	
285	A	RH	1	-3	King B NC	+		
286	F	RH	1	-3	King B NC		no growth on isolation media/liquid culture	
287	B	L	2	-2	King B NC	-		
288	B	RH	5	-2	King B NC	+		
289	B	RH	5	-2	King B NC	+		
290	B	RO	4	-1	King B NC	+		
291	A	RH	4	-3	King B NC	+		
292	B	RO	1	-1	King B NC	+		
293	A	RH	1	-1	King B NC	+		
294	B	RH	1	-1	King B NC	+		
295	B	L	4	-3	King B NC	-	Pseudomonas fluorescens	
296	B	L	4	-3	King B NC	+		
297	B	L	2	0	King B NC	+		
298	B	L	2	0	King B NC	+		
299	B	RH	5	-1	King B NC	-	fungal contamination, lost	

300	B	L	3	-2	King B NC	+		
301	B	L	3	0	King B NC	+		
302	B	L	2	0	King B NC	+		
303	F	L	2	0	King B NC		no growth on isolation media/liquid culture	
304	A	RH	2	-2	King B NC	+		
305	B	RH	2	-1	King B NC	+		389
306	B	RH	2	-1	King B NC	+		
307	B	L	4	0	King B NC	+		
308	B	L	3	0	King B NC	+		
309	B	L	3	0	King B NC	+		
310	B	L	5	-1	King B NC	+		
311	B	RH	5	-1	King B NC	+		
312	F	RH	5	-1	King B NC			
313	B	L	4	-2	King B NC	+		
314	B	L	2	-1	GAC NC	+		421
315	B	L	3	-2	GAC NC	+		317
316	B	L	3	-3	GAC NC	+		
317	B	L	3	-1	GAC NC	+	redundant	315
318	A	L	1	0	GAC NC	+		
319	B	L	1	0	GAC NC	+		
320	B	L	4	-1	GAC NC	+		323
321	A	L	4	-1	GAC NC	+		
322	A	L	5	-2	GAC NC	+		
323	B	L	4	0	GAC NC	+	redundant	320
324	A	RH	5	0	PDA	+		
325	A	RH	5	-2	PDA	+		
326	A	RH	2	0	PDA	+		
327	B	L	4	-2	PDA	+		
328	B	L	2	0	PDA	-	redundant	109
329	B	RH	1	-2	PDA	+		
330	B	RH	5	-3	PDA	+	redundant	200, 201
331	B	L	5	-1	PDA	+		
332	B	L	4	0	PDA	+		
333	F	RH	1	0	PDA			
334	B	RH	1	0	PDA	-		489, 495, 519, 538
335	B	RH	1	0	PDA	+		
336	A	RH	1	0	PDA	+		
337	B	RH	1	-1	PDA	-		
338	B	L	1	-3	SNA	+		
339	B	L	1	-2	SNA	+		
340	B	L	4	-2	SNA	+		
341	B	L	5	-1	SNA	+		

342	B	L	5	-1	SNA	+		
343	B	L	5	-1	SNA	+		
344	B	L	5	-2	SNA	+		
345	B	L	5	-2	SNA	+		
346	A	L	5	-2	SNA	+		
347	B	L	4	-1	SNA	+		
348	B	L	4	-1	SNA	+		
349	B	L	1	0	SNA	+		
350	B	L	1	0	SNA	+		
351	B	L	1	0	SNA	+		
352	B	L	5	0	SNA	+		
353	B	L	5	0	SNA	+		
354	B	L	5	0	SNA	+		
355	B	L	4	0	SNA	+		
356	B	L	4	0	SNA	+		
357	B	L	1	-1	SNA	+		
358	B	L	1	-1	SNA	+		
359	B	L	1	-1	SNA	+		
360	B	RH	2	-2	King B NC	+		
361	B	RH	2	-2	King B NC	-		
362	B	RH	2	-2	King B NC	-		
363	B	RH	4	-2	King B NC	+		
364	A	RH	4	-2	King B NC	+		
365	B	RO	2	-2	King B NC	+		413
367	B	L	4	0	King B NC	+		
368	B	L	4	0	King B NC	-	Pseudomonas fluorescens	
369	B	RH	3	-2	King B NC	+		224, 283, 370, 378
370	B	RH	3	-1	King B NC	+		224, 283, 369, 378
371	B	RH	3	-1	King B NC	+		
372	B	RH	1	-1	King B NC	+		
373	B	L	5	0	King B NC	+		
374	B	L	4	-2	King B NC	+		
375	A	RH	2	-1	King B NC	+		
376	B	RH	1	-3	King B NC	+		
377	B	L	5	-1	King B NC	+		
378	B	L	1	-1	King B NC	+		224, 283, 369, 370
379	B	L	2	0	King B NC	-		
380	B	RH	5	-1	King B NC	+		
381	B	L	5	-2	King B NC	-		
382	A	L	5	-2	King B NC	-		
383	B	RO	5	0	King B NC	+		
384	B	RH	5	-3	King B NC	+		

385	B	RH	5	-3	King B NC	-		
386	B	RH	3	-3	King B NC	+		
387	B	RH	3	-3	King B NC	+		
388	B	RH	3	-1	ISP2 NC	+		
389	B	RH	2	-1	ISP2 NC	+	redundant	305
390	B	L	5	-1	ISP2 NC	+		
391	B	L	3	0	ISP2 NC	+		
392	B	L	4	0	ISP2 NC	+		
393	B	L	2	0	ISP2 NC	+		
394	A	RH	1	0	ISP2 NC	+		
395	B	RH	1	0	ISP2 NC	+		
396	A	RH	1	0	ISP2 NC	+		
397	B	L	1	0	ISP2 NC	-		
398	B	L	1	0	ISP2 NC	+		
399	B	L	2	-2	ISP2 NC	+		
400	B	RH	5	0	ISP2 NC	+		
401	F	RH	5	0	ISP2 NC			
402	A	RH	5	0	ISP2 NC	+		
403	A	RH	3	0	ISP2 NC	+		
404	A	RO	5	-3	ISP2 NC	+		
405	B	RO	5	-3	ISP2 NC	+		
406	B	L	4	-1	ISP2 NC	+		
407	B	L	4	-1	ISP2 NC	+		
408	A	RH	2	0	ISP2 NC	+		
409	B	RH	2	0	ISP2 NC	+		
410	B	RH	1	-1	ISP2 NC	+		
411	A	RH	1	-1	ISP2 NC	+		
412	B	L	3	-1	ISP2 NC	+		
413	B	RO	2	-1	ISP2 NC	+	redundant	365
414	B	L	5	-2	PDA	+	redundant	250
415	A	RH	1	-2	ISP2 NC	+		
416	B	RH	5	-2	ISP2 NC	+		
417a	B	RH	5	-2	ISP2 NC	+		
417b	B	RH	5	-2	ISP2 NC	-		
418	B	L	4	-2	GAC	+	redundant	144
419	F	RH	5	-2	GAC			
420	B	RH	5	-2	GAC	+		
421	B	L	2	-2	GAC	+	redundant	314
422	A	L	4	0	GAC	+		
423	B	L	1	-1	GAC	+		
424	B	L	1	-1	GAC	-	redundant	153
425	B	L	1	-1	GAC	-		

426	B	L	5	-1	GAC	-	Epichloe like
427	F	RH	5	-1	GAC		
428	B	RH	3	-2	King B NC	+	yellowish, small, regular, shiny
429	B	L	3	0	King B NC	-	
430	A	RH	3	-1	King B NC	+	
431	A	RH	3	-1	King B NC	-	
432	A	RH	4	-1	King B NC	+	
433	A	RH	1	-1	King B NC	+	
434	B	RH	1	-1	ISP2 NC	+	
435	Am	RH	1	-2	ISP2 NC	-	
436	Am	RH	1	-2	ISP2 NC	+	
437	A	RH	1	-2	ISP2 NC	+	
438	A	RH	1	0	ISP2 NC	+	
439	B	L	2	-1	ISP2 NC	+	
440	A	RH	2	-1	ISP2 NC	+	
441	A	RH	2	0	ISP2 NC	+	
442	B	L	3	0	ISP2 NC	+	
443	A	RH	3	0	ISP2 NC	-	
444	A	RH	3	-1	ISP2 NC	-	
445	A	RH	4	-1	ISP2 NC	+	
446	B	L	4	0	ISP2 NC	+	
447	F	RH	5	-2	ISP2 NC		
448	B	RH	5	-1	ISP2 NC	+	white-yellow, small, shiny, regular
449	A	L	5	0	ISP2 NC	+	yellow, dull, cup shaped, thick ridges, bottom flat, irregular
450	A	L	5	0	ISP2 NC	-	yellow brownish, dull, brain shaped
451	A	L	5	0	ISP2 NC	-	yellow, cup shaped, central ridges, large, reg.
452	B	L	5	0	King B NC	+	white-yellow, dull, regular
453	F	RH	5	0	King B NC		
454	B	RH	5	-2	King B NC	+	yellowish transparent, shiny, reg.,
455	B	RH	5	-2	King B NC	+	bright yellow, shiny, regular
456	B	L	5	0	King B NC	+	bright yellow, concentr, reg, shiny
457	B	L	2	0	King B NC	+	small, orange, concentr., shiny, regular
458	A	RH	2	-1	King B NC	+	large, yellow transparent, centr, ridges radial, builds crystals
459	A	RH	2	-2	King B NC	+	small, ivory, shiny, digests agar
460	B	RH	2	0	HV NC	+	large, brownish, concentr., regular, shiny
461	B	RH	2	0	HV NC	+	large, ivory center, conc., regular, shiny
462	B	RH	2	0	HV NC	+	yellow, dull, reg,
463	B	RH	3	-1	HV NC	+	brown?, shiny, regular
464	B	RH	3	-1	HV NC	-	large
465	B	RO	5	-2	HV NC	+	large, conc., regular, brown, shiny
466	B	RH	4	-2	HV NC	+	colony appr. 3cm, hyphae on edge
467	B	L	5	-1	HV NC	+	small, yellow, shiny, regular

468	B	L	5	-1	HV NC	-	orange, dull, regular	
469	B	L	5	-1	HV NC	+	orange-white, shiny, regular	
470	B	L	5	-1	HV NC	+	ivory-brown, large, shiny, regular	
471	B	L	5	-1	HV NC	+	yellow-white, large, shiny, regular	
472	B	L	1	-1	HV NC	+	ivory, large, shiny, regular	
473	B	L	1	-1	HV NC	-	orange, small, shiny, regular	
474	F	RH	4	0	HV NC			
475	B	L	4	-1	HV NC	+	ivory transparent, large, irregular, shiny	
476	B	L	4	-1	HV NC	+	orange, small, shiny, regular	
477	B	L	4	-1	HV NC	+	white, large, shiny, regular	
478	B	L	4	-1	HV NC	+	dark brown, small, shiny	
479	B	RO	4	0	HV NC	+	ivory, center brown, 3D, irregular, dull	
480	B	RH	4	-1	HV NC	-	colony appr. 3cm	
481	B	RH	4	-1	HV NC	+	dark brown, regular, dull	
482	F	RH	5	-1	HV NC		sporuliert, Ø ca. 7mm	
483	B	RH	5	-1	HV NC	+	large, yellowish, 3D, regular, dull	
484	A	RH	5	-1	HV NC	-	very small colonies, contamination, also on LN2 RH-3	
485	B	L	5	0	HV NC	+	large, white, shiny, regular	
486	B	L	1	0	HV NC	+	small, yellow, shiny, regular	
487	F	RH	1	-1	PDA		grey	
488	F	RH	1	-1	PDA		rose	
489	B	RH	1	-3	PDA	+	deep orange, small, regular, shiny	334, 495, 519, 538
490	A	RH	1	-3	PDA	-	yellow, reg., shiny, ring with radial ridges	
491	B	RH	1	-3	PDA	+	white-transp., reg. Small, shiny	
492	A	RH	1	-2	PDA	+	yellow ring, irreg., 3D, dull	
493	B	L	1	-1	PDA	-	yellow, small, reg.,	
494	B	L	1	-1	PDA	-	orange, small, reg.,	
495	B	L	2	-1	PDA	-	deep orange, regular, shiny	334, 489, 516, 519, 538
496	B	L	2	0	PDA	-	salmon, reg., shiny	
497	B	L	2	0	PDA	-	white-samlon, runny, shiny	
498	B	RH	2	-3	PDA	-	white, large, shiny, regular, runny	
499	A	RH	2	0	PDA	+	white, irreg., dull, 3D, flower like	
500	A	RH	2	0	PDA	+	white, flat, irreg., 3D, large	
501	A	RH	2	0	PDA	-	salmon, 3D, reg., flower like, dull	
502	B	RH	2	0	PDA	-	yellow-transp., reg. Shiny	
503	B	L	3	-1	PDA	+	pink, tiny, regular	
504	F	RH	3	0	PDA		white, irreg., bact. Overgrowth	
505	Am	RH	3	0	PDA	+	white Am, 3D, reg., digest agar	
506	A	RH	3	0	PDA	-	ivory, waxy, reg, digests agar, diameter creek	
507	A	RH	3	0	PDA	-	dirty ivory, waxy, reg, digests agar	
508	A	RH	3	0	PDA	-	ivory, 3D, irreg.,	
509	F	RH	3	-1	PDA		white with dark center	

510	A	RH	3	-2	PDA	-	white waxy, no 3D, reg	
511	F	RH	4	-1	PDA		rose-grey, bacteria with antifungal effect	
512	F	RH	4	-1	PDA		white, small	
513	F	RH	4	0	PDA		grey regular, digests agar	
514	A	L	4	0	PDA	-	yellow gallerty, irregular, dull	
515	A	L	4	0	PDA	-	yellow gallerty, irregular, dull, cup shaped	
516	B	L	4	0	PDA	-	deep orange, small, regular, shiny	334, 489, 495, 519, 538
517	B	RH	5	0	PDA	-	dil 0 mostly with fungus, dil 123 mostly with bact., no fungus!	
518	A	RH	5	0	PDA	-	ivory, waxy, reg, ring 3D	
519	B	RH	5	-2	PDA	-	deep orange, small, regular, shiny	334, 489, 495, 516, 538
520	A	L	5	-1	PDA	-	yellow- white, irreg., 3D, dull, gallerty	
521	A	L	5	-2	PDA	+	yellow, 3D, reg., dull, large, digests agar	
522	B	L	4	-1	SNA	+	rose-white, small, reg, shiny	
523	A	L	1	-1	SNA	+	yellow-white, transp, 3D, reg.	
524	B	RH	2	-2	SNA	+	salmon-transp., shiny, reg	
525	F	L	2	0	SNA		Trichoderma sp.	
526	A	L	5	-1	SNA	+	ivory, small, 3D, irreg	
527	A	L	5	-1	SNA	+	ivory, small, reg	
528	A	L	1	-1	GAC NC	+	yellow, gallerty, reg., large	
528b	B	L	1	-1	SNA	+		
529	B	L	3	0	GAC NC	+	rose, small, shiny reg	
530	F	RH	3	0	GAC NC		white with grey center, small	
531	B	RH	3	0	GAC NC	+	ivory, reg., shiny	
532	B	RH	5	-1	GAC NC	+	like 529	
533	B	RH	5	-2	GAC NC	+	deep yellow, dull, reg	
534	A	RH	5	-2	GAC NC	+	deep yellow, 3D, reg, ring	
535	A	RH	5	-2	GAC NC	+	tiny, irreg, ivory	
536	B	RH	5	-2	GAC NC	+	transparent, large, very thin, reg	
537	A	L	5	0	GAC NC	+	yellow-transparent, gallerty, irreg., ear	
538	B	L	5	0	GAC NC	+	deep orange, small, regular, shiny	334, 489, 495, 516, 519
539	B	L	5	-2	GAC NC	-	deep yellow, small, regular, shiny	554
540	A	L	5	-1	GAC NC	+	yellow-gallerty, 3D, ring, reg	
541	A	L	1	0	GAC NC	+	yellow-gallerty, 3D, ring, reg	
542	B	RH	2	-2	GAC NC	+	white, large, reg., shiny	
543	B	RH	2	-2	GAC NC	+	white-transp., medium, reg., shiny	
544	B	RH	2	-3	GAC NC	-	ivory reg., shiny	lost
545	B	L	2	-2	GAC NC	+	deep yellow, small, regular, shiny	539
546	B	RH	1	0	ISP2 NC	-	white, flat, large, curly appearance, like 517	
547	B	RH	1	0	ISP2 NC	-	white, flat, large, curly appearance, like 517	
548	A	RH	1	-1	ISP2 NC	-	tiny, white, 3D, maybe irreg,	
549	A	RH	1	-1	ISP2 NC	+	white, thin, large, 3D ridges	
550	B	RH	4	-1	ISP2 NC	+	ivory, large, flat, dull, irreg, avoidance of gram-neg. twards 550	

551	B	RH	4	0	ISP2 NC	-	white, flat, large, curly appearance, like 517	
552	B	RH	4	0	ISP2 NC	-	white, flat, large, curly appearance, like 517	
553	B	RH	4	0	ISP2 NC	-	white, flat, large, curly appearance, like 517	
554	B	RH	1	-2	King B NC	+	rose, small, reg, dull	
555	B	RO	1	-1	King B NC	+	white large curly	
556	B	RH	1	-1	King B NC	-	white, flat, large, curly appearance, like 517	
557	B	RO	1	0	King B NC	+	white large curly	
558a	B	RH	2	-1	King B NC	+	white large curly	
558b	A	RH	2	-1	King B NC	+	ivory, 3D, reg, dull	
559	B	L	3	-2	King B NC	+	deep yellow, reg. Shiny	
560	B	RO	3	-1	King B NC	+	yellow-wh, reg, shiny	
561	B	L	3	0	King B NC	+	white large curly	
562	B	RH	5	-3	King B NC	+	deep yellow, large, shiny, reg	
563	B	RO	5	-2	King B NC	+	yellow-wh, reg, small, shiny	
564	B	RO	5	-1	King B NC	+	ivory, irreg, dull	
565	B	RO	5	-1	King B NC	+	ivory, large, reg, dull, flat	
566	A	L	5	-2	King B NC	+	organge, 3D, reg, dull	
567	B	L	3	0	King B NC	+	white, large, reg., shiny	
568	B	L	3	0	ISP2 NC	+	white pink, dull, reg	
569	B	L	3	-1	King B NC	+	deep orange, dull, reg	
570	B	L	3	-2	ISP2 NC	-	ivory, reg., shiny	
571	A	RH	3	0	King B NC	+	green-grey, 3D	
572	A	RH	3	0	King B NC	+	white border, dark center, 3D	
573	A	RH	3	-1	King B NC	+	white, 3D, reg, ridges	
574	A	RH	1	-1	ISP2 NC	+	OS: white, US: brown, irreg, ridges	
575	A	RH	1	-1	ISP2 NC	-	orange-transp. 3D, irreg	
576	A	RH	1	-1	ISP2 NC	+	orange-wh, gallerty, reg	
577	B	RH	1	-3	ISP2 NC	+	pink, tiny, regular	
578	A	RH	1	0	ISP2 NC	+	yellow-transparent, gallerty, irreg, brain like	
579	B	L	2	0	ISP2 NC	-	brown, shiny, regular	
580	A	RH	2	-2	ISP2 NC	+	2 actinos growing together	
581	A	RH	2	0	ISP2 NC	+	white, ear, disattaches from surface, irreg	
582	B	RH	1	-2	ISP2 NC	+	grey-wh, concentr., reg, 3D	
583	B	RH	1	-2	ISP2 NC	+	pink, tiny, regular	
584	A	L	5	0	ISP2 NC	+	yellow, gallerty, irreg	
585	A	L	5	0	ISP2 NC	+	yellow-transparent, gallerty, irreg, brain like	
586b	B	RH	5	0	HV NC	+	no. assigned to two strains (concentr., reg., spores grey; pink, reg, small)	WH 2 strains
586a	B	RH	5	0	HV NC	+	no. assigned to two strains (concentr., reg., spores grey; pink, reg, small)	
587	B	RH	1	-1	HV NC	+	brown, large, reg, dull	
588	B	RH	1	-1	HV NC	+	beige, large, dull, reg	
589	B	RH	1	-1	HV NC	+	white large curly	
590	A	RH	2	0	HV NC	+	white, reg	

591	B	L	3	0	HV NC	-	pink, tiny, regular	does not grow in TSB
592	B	L	4	0	HV NC	+	yellow, reg., shiny	
593	A	RH	5	0	HV NC	+	white, small, reg	
594	B	RH	5	0	HV NC	+	white, small, reg, shiny	
595	B	L	3	-1	HV NC	+	yellow, large, dull, reg	
596	F	RH	4	0	PDA		large with black cleistothecia	
597	A	L	5	0	PDA	-	ivory, reg, 3D	
598	A	L	5	0	PDA	-	yellow-wh, ring, 3D	
599	B	RH	5	-3	PDA	+	yellow-orange, 3D, large, reg	
600	B	RH	5	-2	PDA	+	pink, tiny, regular	
601	F	RH	5	-1	PDA		white	
602	A	L	5	-1	PDA	+	ivory, ring, 3D, gallerty, reg	
603	B	L	4	-1	PDA	+	pink, tiny, regular	
604	B	RH	4	-2	PDA	+	large, brown, dull, reg	
605	B	RH	4	-3	PDA	+	ivory-transp., reg, small	
606	A	RH	3	0	PDA	+	small white, with grey center	
607	A	RH	3	0	PDA	+	small, less white	
608	A	RO	3	0	PDA	+	yellow-white, 3D, ring, irreg	
609	B	RH	3	-2	PDA	+	wh-transp., small reg, shiny	
610	A	RH	3	-3	PDA	+	rose, small, reg	
611	B	RH	2	-3	PDA	+	white, reg, shiny	
612	F	RH	2	-1	PDA		small, black	
613	B	L	2	-1	PDA	+	deep yellow, shiny, reg	
614	F	L	2	-3	PDA		white, small	
615	B	RH	1	-2	PDA	+	rose, transp., reg, shiny	
616	B	RH	1	-3	PDA	+	orange, dull, irreg	
617	A	L	1	0	PDA	-	large, ivory, flat, dull	
618	B	RH	5	-2	SNA	+	white transp., shiny, large, runny	
619	B	RH	5	-3	PDA	+	rose, reg, shiny	
620	B	RH	5	-3	PDA	+	yellow white, shiny, reg, filamentuos tow. 619, runny	
621	F	RH	3	0	SNA		white	
622	F	RH	3	-1	SNA		white with grey center	
623	A	L	1	0	SNA	+	ivory, reg, dull, with 3 parallel cracks	523
624	B	RH	3	-2	GAC NC	+	rose, reg, shiny, small	
625	B	RH	3	-1	GAC NC	+	ivory, reg, small, shiny	
626	B	RH	3	-1	GAC NC	+	white-transp, reg, shiny	
627	A	L	5	0	GAC NC	+	yellow-gallerty, 3D, reg	
628	A	RH	1	-1	GAC NC	+	ivory, 3D	
629	B	RH	1	-2	GAC NC	-	transp., large, flat, dull, reg	
630	B	RH	1	-2	GAC NC	-	white large curly	
631	B	RH	1	-2	GAC NC	-	rose, small, reg, shiny	
632	B	RO	2	-2	GAC NC	+	white-tansp, reg, small	

633	B	L	2	0	GAC NC	+	white-tansp, reg, small
634	B	RH	1	-3	GAC NC	-	deep yellow, small, regular, shiny
635	F	RH	2	0	GAC NC		dark
636	F	RH	2	0	GAC NC		grey
637	B	RH	4	-1	GAC NC	+	white, reg, dull
638	B	RH	4	-2	GAC NC	-	white large curly
639	B	RH	4	-3	GAC NC	+	beige, small, reg
640	Am	RH	5	0	ISP2 NC	+	large, 3D, reg
641	A	RH	5	0	ISP2 NC	+	yellow, small, reg., 3D
642	Am	RH	5	0	ISP2 NC	+	yellow, 3D, reg,
643	Am	RH	5	0	ISP2 NC	+	ivory, grainy, 3D, white top, ridges
644	Am	RH	5	0	ISP2 NC	+	grey, 3D, ridges
645	Am	RH	5	0	ISP2 NC	+	white, 3D, cup, irreg
646	Am	RH	5	0	ISP2 NC	+	white-grey, large, 3D, irreg
647	Am	RH	4	0	ISP2 NC	+	grey-white, concentr, reg
648	Am	RH	4	0	ISP2 NC	+	grey-white, reg
649	A	RH	4	-1	ISP2 NC	+	yellow-wh, irreg, ridges, 3D
650	B	RH	4	-1	ISP2 NC	+	ivory, large, flat, dull, irreg, ev. Pseudomonas
651	Am	RH	2	0	ISP2 NC	+	grey, 3d, reg
652	Am	RH	2	0	ISP2 NC	+	white, 3D, reg, ridges
653	Am	RH	2	0	ISP2 NC	+	grey-white, 3D, irreg
654	Am	RH	2	0	ISP2 NC	+	white ridges, 3D, irreg
655a	B	RH	2	0	ISP2 NC	+	
655b	Am	RH	2	0	ISP2 NC	+	white cup, 3D, irreg
656	Am	RH	1	0	ISP2 NC	+	grey regular
657	Am	RH	1	0	ISP2 NC	-	grey regular
658	A	RH	1	0	ISP2 NC	+	yellow-wh, irreg, ridges, 3D
659a	A	RH	1	0	ISP2 NC	-	yellow-wh, reg
659b	B	RH	1	0	ISP2 NC	-	
660	A	RH	1	-1	ISP2 NC	+	grey white, concentr.
661	A	RH	1	-2	ISP2 NC	+	white, 3D,
662	A	RH	1	-2	ISP2 NC	+	grey, 3D, cooc with 435
663	B	RH	1	-2	ISP2 NC	+	white, ring
664	B	RH	1	-2	ISP2 NC	+	pink, tiny, regular
665	A	RH	1	-2	ISP2 NC	+	yellow, gallerty, tiny, reg
666	B	L	3	0	ISP2 NC	+	pink, tiny, regular
667	Am	RH	3	0	ISP2 NC	+	grey-wh, flat, reg
668	Am	RH	3	0	ISP2 NC	+	grey, 3D, reg
669	Am	RH	3	0	ISP2 NC	+	light grey, 3D, vulcano, reg
670	Am	RH	3	0	ISP2 NC	+	grey, 3D, ridges and exudate drops
671	Am	RH	3	-1	ISP2 NC	+	white-grey, 3D reg
672	Am	RH	3	-1	ISP2 NC	+	dark-grey, 3D reg

673	Am	RH	3	-1	ISP2 NC	+	white, 3D, reg
674	A	RH	3	-3	King B NC	+	ivory, no aerial mycelia, reg
675	F	RO	2	0	King B NC		white-grey, small
676	B	RH	2	-2	King B NC	+	white, flat, grainy, reg
677	A	RH	1	-1	King B NC	+	ivory, transp., 3D, reg
678	B	RH	1	-1	King B NC	+	ivory, transp., 3D, reg
679	A	RH	4	-1	PDA	+	yellow, gallerty, reg, small
680	A	L	5	-2	PDA	+	yellow gallerty, large, 3D, irreg
681b	Am	RH	3	-2	PDA	+	brown with withe border, reg, 3D
682	A	RH	1	-2	GAC NC	-	brown, reg, small, dull
683	F	RH	4	0	GAC NC		black
684	A	RH	2	-1	GAC NC	+	transp, irreg, small, flat
685	A	RH	3	0	GAC NC	+	dark-grey, 3D, reg, DA
686	A	RH	3	0	GAC NC	+	grey, small, reg
687	B	L	1	-1	King B NC	+	transp., shiny, concentric
688	Am	RH	5	-3	ISP2 NC	+	yellow, 3D, white top grey spores, irreg
689	Am	RH	5	0	ISP2 NC	+	light grey, 1 ridge through center, reg
690	Am	RH	5	0	ISP2 NC	+	yellow top, white bottom, irreg
691	Am	RH	2	0	ISP2 NC	-	white small reg
692	Am	RH	1	-2	ISP2 NC	+	grey-green, flat, regular
693	Am	RH	1	-2	ISP2 NC	+	yellow vulcano, hole in the top, reg, 3D
694	Am	RH	3	?	ISP2 NC	+	grey, concentr., reg
695	F	RH	3	?	ISP2 NC		grey
696	F	RO	2	-3	King B NC		white grey, small, reg
697	F	L	5	0	PDA		brown grey, whith huge dark center
698	B	RH	1	-2	PDA	+	rose shiny, reg
699	A	RH	1	-2	PDA	+	yellow, 3D, hole in middle, irreg
700	A	RH	2	0	PDA	+	yellow white, irreg, disattached to Agar
701	B	RH	3	-2	GAC NC	-	white, small, shiny, reg
702	B	RH	5	-3	GAC NC	+	rose-orange, large, shiny, reg
703	B	RH	5	-3	GAC NC	+	rose, small, flat, dull, reg
704	Am	RH	3	0	HV NC	+	white, reg
705	A	RH	4	0	HV NC	-	orange stick, grows vertically
706	B	RH	2	0	HV NC	-	dark brown, shiny, reg
707a	B	RH	1	-1	HV NC	+	pink, reg, shiny
707b	B	RH	1	-1	HV NC	+	white curly
708a	B	RH	1	-1	HV NC	+	pink, reg, dull concentric
708b	B	RH	1	-1	HV NC	+	white curly
709	B	RH	1	0	HV NC	-	white curly
710	A	RH	5	0	ISP2 NC	+	white reg
711	A	RH	5	0	ISP2 NC	+	grey-brown, reg, crack 3 pointed
712	A	RH	5	0	ISP2 NC	+	grey yellow, irreg

713	Am	RH	1	0	ISP2 NC	-	white
714	Am	RH	1	-1	ISP2 NC	+	white rose, irreg
715	Am	RH	1	-2	ISP2 NC	-	white rose
716	Am	RH	4	0	ISP2 NC	+	white, concentr., reg
717	Am	L	3	0	ISP2 NC	+	brown, dull, reg
718	A	L	3	0	ISP2 NC	+	light rose, reg, dull
719	B	RH	3	-2	ISP2 NC	+	light rose, irreg, 3D, flat
720	B	RH	3	-2	ISP2 NC	+	white, irreg, 3D, flat
721	B	RH	3	-1	ISP2 NC	+	pink, transp, flat
722	B	RH	3	-1	ISP2 NC	+	orange, small, reg, dull
723	Am	RH	5	0	ISP2 NC	+	white, 3D, vulcano, reg
724	A	L	5	0	ISP2 NC	+	white-yellow, gelatious, tansp, irreg
725	B	RH	1	-2	HV NC	+	rose, large, concentr, reg, shiny
726	B	RH	2	0	HV NC	+	brown, concentr, 3D, dull, reg
727	Am	RH	2	0	HV NC	+	white, reg
728	B	L	4	0	HV NC	+	rose concentr, reg, shiny
729	Am	RH	5	-3	HV NC	+	white, thin, large, reg
730	F	RH	5	0	HV NC		white-green, large
731	Am	RH	5	0	HV NC	+	white, reg
732	B	RH	4	-1	GAC NC	-	white, curly, tightly knitt
733	F	RO	2	-2	GAC NC		golden, daily rings
734	B	RO	2	-1	GAC NC	+	yellow, shiny, reg
735	B	RH	1	-1	GAC NC	+	rose, 3D ridges, reg, dull
736	B	RH	1	-3	GAC NC	+	transp, large, flat
737	B	RH	1	-1	GAC NC	+	rose, small, concentr, reg
738	A	RH	1	-3	GAC NC	+	rose, tiny, irreg, dull
739	A	RH	2	-3	GAC NC	+	rose, tiny, irreg, dull
740	A	RH	3	0	GAC NC	+	red-brown, concentr, dull, reg
741	A	RH	2	-1	GAC NC	+	rose, irreg, dull, 3D
742	B	RH	4	-1	King B NC	-	transp, reg, concentr, flat
743	A	RO	4	-2	PDA	+	orange, 3D
744	A	RO	4	-2	PDA	+	orange, 3D, reg
745	A	RH	4	-2	PDA	+	zinnober red, reg
746	A	L	4	-3	PDA	+	orange, large, 3D, irreg
747	B	RH	3	-3	PDA	-	yellow-white, shiny reg
748	B	RO	2	-1	PDA	+	ivory, shiny, reg
749	B	RO	2	-2	PDA	+	orange, dull, regular
750	A	RO	3	-1	King B NC	+	dark orange, small, reg, agar digest
751	A	RO	3	-1	King B NC	+	orange, 3D, irreg
752	B	RO	3	-1	King B NC	+	orange, concentr, dull, reg
753	A	RO	4	0	King B NC	+	orange, dull, reg, agar digest
754	A	RO	4	-1	King B NC	-	dark orange, ring, reg

755	A	RH	4	-3	King B NC	+	orange, dull, agar eating
756	A	RO	5	-1	King B NC	+	orange, 3D, irreg
757	A	RO	2	-2	King B NC	+	orange, dull, reg
758	A	RH	1	-2	King B NC	-	ivory, large, flat, dull, 3D
759	Am	RH	2	0	ISP2 NC	-	grey, reg
760	A	L	2	-1	ISP2 NC	+	orange, small, dull, reg
761	A	RH	1	-3	ISP2 NC	+	orange, tiny, reg, dull
762	A	RH	5	0	ISP2 NC	+	brown, tiny, reg
763	A	RH	5	0	ISP2 NC	+	brown-red, tiny, reg
764a	A	RH	4	0	ISP2 NC	-	salmon, hilly, irreg, 3D
764a	B	RH	4	0	ISP2 NC	-	
765	Am	RH	4	0	ISP2 NC	-	grey, reg
766	A	RH	4	0	ISP2 NC	-	yellow-white, irreg, 3D
767	A	RO	4	0	King B NC	+	yellow orange, reg, 3D
768	B	L	4	0	King B NC	+	yellow, shiny, reg
769	B	RO	1	0	King B NC	-	curly white
770	A	RH	1	-1	King B NC	-	ivory, reg, 3D, dull
771	Am	RH	1	0	King B NC	+	white, reg
772	A	RO	3	-1	King B NC	+	orange, small, dull, reg
773	A	RH	3	-3	King B NC	+	orange, small, dull
774	B	RH	3	-2	King B NC	+	curly white
775	F	RH	2	0	PDA		fungus white, stains bacteria dark red
776	B	RH	2	0	PDA	-	dark red, reg
777	F	L	1	-1	PDA		small, dark
778	B	L	5	0	SNA	-	transparent, irreg, grainy centers
779	A	L	5	-2	GAC NC	+	orange, large, flat, reg
780	A	RO	2	-2	GAC NC	+	orange, irreg
781	A	RH	2	-1	GAC NC	+	yellow, gelatious, 3D, reg
782	A	RH	4	-3	GAC NC	+	orange, small, dull, reg
783	A	RH	4	0	GAC NC	+	white, 3D, gelatinous, reg
784	A	RH	1	-2	HV NC	-	yellow, gelatious, 3D
785	Am	RH	1	0	HV NC	+	white, reg
786	B	L	3	-1	HV NC	+	rose, shiny, reg
787a	B	RH	4	-2	HV NC	-	brown, shiny, reg
787b	B	RH	4	-2	HV NC	-	
788	Am	RH	5	0	HV NC	+	grey, reg
789	Am	RH	5	0	HV NC	-	black, reg
790	A	RH	1	-3	ISP2 NC	+	dirty yellow, dull, irreg, agar digest
791	B	L	4	-1	ISP2 NC	+	grey, dull, reg
792	B	RO	2	0	ISP2 NC	+	white, 3D ridges, irreg, dull
793	Am	RH	5	0	ISP2 NC	-	grey, 3D, irreg
794	A	RO	4	0	King B NC	+	orange, 3D, irreg

795	F	RH	1	-2	King B NC		white grey, irreg
796	A	L	1	0	King B NC	+	ivory, 3D, dull, irreg
797	F	RH	5	-1	PDA		white, very large in 1 week, dense mycelia
798	B	L	5	-1	PDA	-	white, concentr., reg, shiny
799	F	RH	4	-2	PDA		black, hyphae mostly under agar
800	A	RO	4	-2	PDA	+	orange, irreg
801	A	RO	4	-2	PDA	+	orange, large, irreg
802	A	RO	4	-2	PDA	-	deep orange, irreg, 3D
803	A	RO	4	-2	PDA	+	orange, reg
804	A	RH	2	-1	GAC NC	+	brown, reg, dull
805	A	RH	3	0	GAC NC	+	ivory, tiny, 3D
806	A	RH	3	-1	GAC NC	+	light orange, reg, dull, agar digest
807	Am	RH	5	0	GAC NC	+	white
808	crystal	L	5	-2	GAC NC	+	parallel raphid like crystals, in rays from center, diam. Ca. 7mm
809	F	RH	2	0	HV NC		black fungus, elevates agar in center
810	B	L	1	0	HV NC	-	dark, flat, dull
811	Am	RH	3	0	HV NC	-	white, concentr., reg
812	Am	RH	4	-1	HV NC	-	white, concentr., reg
813	B	RH	4	-3	ISP2 NC	+	grey, reg, shiny
814	B	RH	1	0	King B NC	+	salmon, dull, irreg
815	B	RO	4	-2	King B NC	+	white, curly
816	B	RH	3	-2	King B NC	+	white, curly
817	F	RH	1	-3	PDA		penicillium like, underside orange, grows deep into agar
818	Am	RH	1	-1	GAC NC	+	red-brown, small, reg
819	Am	RH	1	-1	GAC NC	+	salmon, small, reg
820	Am	RH	1	-1	GAC NC	+	salmon, agar digest, reg
821	Am	RH	1	-1	GAC NC	+	ivory, small, reg
822	Am	L	2	-1	GAC NC	+	orange-red, reg
823	Am	RH	5	0	GAC NC	+	pink, 3D, vulcano, reg
824	Am	RH	5	0	GAC NC	+	white, large, reg
825	Am	RH	3	-1	GAC NC	-	red-brown, small, reg
826	B	RH	4	-1	GAC NC	-	white, extremely curly
827	Am	RH	3	0	HV NC	-	white, concentr, reg
828	Am	RH	1	0	HV NC	-	white, reg
829	B	L	1	-1	GAC NC	+	
830	Alga	L	4	0	PDA	+	dark green, shiny (blue-green), regular