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DIPLOMARBEIT

Hairy roots of *Peucedanum ostruthium* (L.) Koch:
Establishment and HPLC analysis

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Abbreviations

MS	Basal medium after Murashige and Skoog (1962)
½ MS	Half-strength MS medium
B5	B5 medium after Gamborg et al. (1968)
BAP	6-benzylaminopurine
GA3	Gibberellic acid
YMB	Yeast mannitol broth
T1-	*Agrobacterium rhizogenes* strain TR 105 and number of infection
A1-	*Agrobacterium rhizogenes* strain ATCC 15834 and number of infection
L1-	*Agrobacterium rhizogenes* strain LBA 9402 and number of infection
-HM	Hairy roots grown in ½ MS medium
-MS	Hairy roots grown in MS medium
-B5	Hairy roots grown in B5 medium

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1. Introduction

1.1. *Peucedanum ostruthium* (L.) Koch

Peucedanum ostruthium (L.) Koch (Koch 1824), also called masterwort (German: Meisterwurz), is a species within the Apiaceae family, as are e.g. plants like carrot or herbs like dill or fennel. Masterwort is widely spread in the alpine and pre-alpine regions of the Alps, but is also found outside of Europe. It is a perennial herb and its average height ranges from 30 cm to 100 cm. The rhizome is stout and branched, the stems are terete, striate, hollow, simple or branched above. The leaves are grass-green, glabrous or hirsute at the bottom and pilose at the leaf margin. Lower leaves are ranging in size up to 30 cm long by 34 cm wide, they are usually 2-ternate and triangular in their outlines. The lobes usually measure 50-100 mm × 40-70 mm and show ovate to lanceolate, acuminate and irregular dentate forms. Middle lobes are 3-lobed and lateral lobes sometimes are 2-lobed (Tutin et al. 1968). The large umbels have up to 50 rays bearing small reddish or white flowers. The fruits are 4 mm to 5 mm circular-shaped achens, pale-white with a brownish disk. *Peucedanum ostruthium* flowers from June to August (Hegi 1965).
This plant prefers humid lime soil at altitudes of 1400 m to 2700 m above sea level in habitats such as mountain meadows, woods, cirque, tall herbaceous vegetations, rocky places and stream banks (Hegi 1965).

Masterwort rhizomes are used as a herbal drug in traditional Austrian medicine for bitters, liquors and teas (Gerlach et al. 2006). *"Peucedanum (officinale)"* is the Latin-Greek name of the plant referring to the bitter taste of the rhizome used for medical purposes. *"Ostruthium"* (meaning "useful", "lucky") was added to the name in the Middle Ages. Masterwort was not known to ancient authors, but was used from 1560 throughout the 19th century as a medicinal plant. It was employed to cure bronchial catarrh, asthma, pestilent illnesses, epilepsy and fever, both in humans and animals (Düll and Kutzelnigg 2011). People in those times believed that masterwort

protected against witchcraft (Hegi 1965). Figure 1 shows *Peucedanum ostruthium*, the species used within this study, in the gardens of the Department of Pharmacognosy in Vienna.

Figure 1: *Peucedanum ostruthium* (L.) Koch

Coumarins and furanocoumarins are important secondary metabolites in the rhizomes of *P. ostruthium.* Within the Apiaceae and the Rutaceae family, about 1300 coumarins have been identified so far, and the occurrence of specific coumarins is used to distinguish between related species (Curini et al. 2006). For masterwort the most important coumarins and furanocoumarins are: Imperatorin, isoimperatorin, osthol, ostruthin, ostruthol, oxipeucedanin and peucenin (Hadacek et al. 1994). The chemical structures of those focused on in this thesis are shown in figure 2 on page 3.

Coumarins and furanocoumarins show antioxidant, antimycobacterial, anticoagulant, antitumoral, antiviral, antifungal and anti-inflammatory activity

(Garcia-Argaez et al. 2000; Schinkovitz et al. 2003; Genovese et al. 2009; Riveiro et al. 2010; Thuong et al. 2010).

These chemical agents are produced by many plants as a defense mechanism against herbivores (Hänsel and Sticher 2007), are also relevent for phototoxic, mutagenic, cancerogenic and heptatotoxic effects (McKenna 2004; da Silva et al. 2009; Abraham et al. 2010).

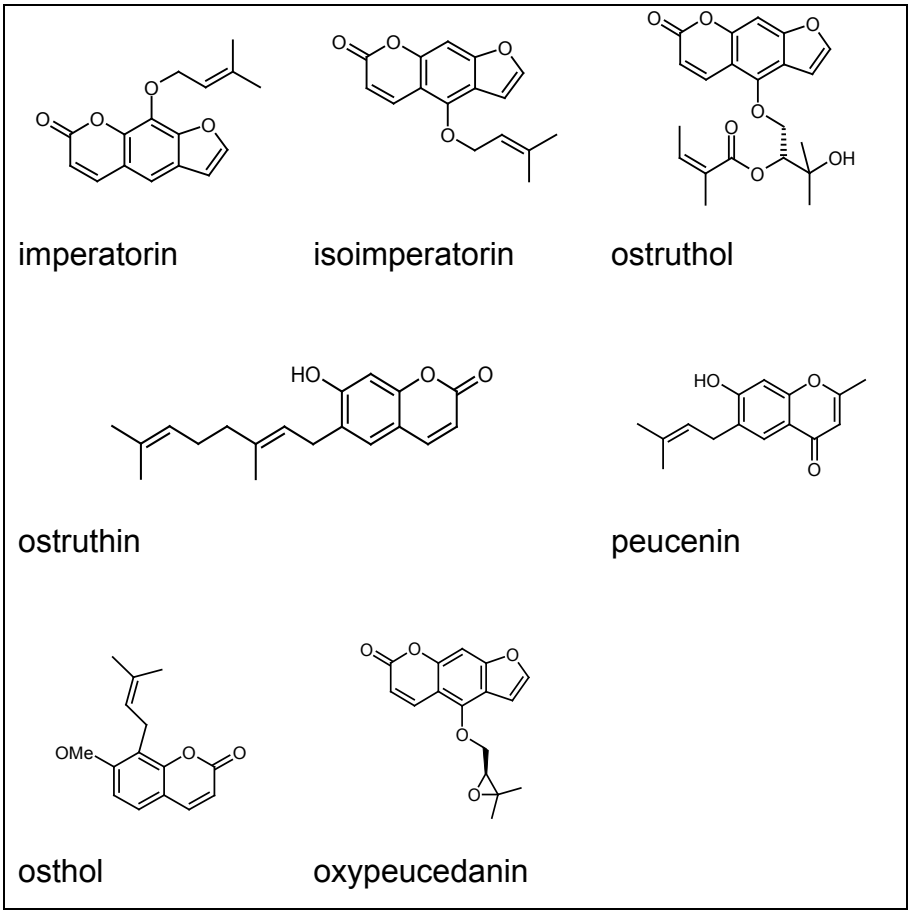

Figure 2: Chemical structures of the most common coumarins and furanocoumarins found in *Peucedanum ostruthium* (Vogl et al. 2011).

Several studies about the pharmaceutical, biological and medical use of *Peucedanum ostruthium* have been published. For instance, it is known that imperatorin and isoimperatorin act as harmful substances on *Spodoptera littoralis* larvae, hindering them in growing, in contrast to ostruthin and osthol, which do not show this effect (Ballesta-Acosta et al. 2008).

Ostruthin in the dichloromethane extract of masterwort rhizomes has an antimycobacterial effect on *Mycobacterium fortuitum.* Imperatorin was also isolated and tested, but showed no activity (Schinkovitz et al. 2003). Gracia-Argaez et al. 2000 isolated imperatorin from *Decatropis bicolor* and examined it in the TPA ear mice model where it developed anti-inflammatory activity on edema. Another finding was that coumarins protect animals against MES-induced seizures due to their anticonvulsant character (Genovese et al. 2009). The antioxidant activity of natural coumarins was confirmed for 17 coumarins isolated from four Korean medical plants (Thuong et al. 2010).
For the treatment of Alzheimer's disease coumarins like ostruthin, imperatorin, ostruthol and peucenin were isolated from a dichloromethane extract of *Peucedanum ostruthium*. They were shown to repress acetylcholinesterase-1 (Urbain et al. 2005). Psoralen (its structure is related to coumarins) is a parent compound of furanocoumarins and is therapeutically used in phytochemotherapy simultaneously with UV-A irradiation (PUVA) for the treatment of skin diseases. However, this therapy carries the risk of skin cancer (McKenna 2004).
Furanocoumarins and coumarins show photosensibilising effects through interaction with the DNA at light wavelengths of 320-410 nm. As a result, transcriptase and polymerase are blocked and cell replication is inhibited (da Silva et al. 2009).

Another important compound of *P. ostruthium* is the essential oil, which the rhizomes contain up to 1.25% of. Sabinene and 4-terpineol are the main substances of 29 compounds that could be identified in the essential oil. In total, 39 compounds were detected by the use of gas chromatography with MS detection and flame ionisation detection (Cisowski et al. 2001).
As the process of dealing with all compounds of *Peucedanum* – among them also the phtalides cnidilide, Z-ligustilide and senkyunolide (Gijbels et al. 1984) – would have been very extensive in time, the main focus of the study was laid on the coumarins and furanocoumarins.

1.2. *Agrobacterium rhizogenes* and hairy roots

Within the bacterial genus *Agrobacterium* there are several strains known to be phytopathogens. *A. tumefaciens* causes crown gall disease, *A. rubi* leads to cane gall disease, and *A. vitis* for example causes galls on grape and other plant species. *A. radiobacter* is a non-virulent bacterium in the *Agrobacterium* genus (Miranda et al. 1992).

Agrobacterium rhizogenes, depicted in figure 3 (old name: *Phytomonas rhizogenes,* latest scientific name: *Rhizobium rhizogenes*) is a Gram-negative soil bacterium (Conn 1942) and was identified as the origin of the so-called hairy-root disease in the first third of the 20th century (Riker et al. 1930; Hildebrand 1934). Numerous, i.e. more than 450, different plant families and genera are known to form hairy roots following infection with this bacterium.

Recent studies on the comparative analyses of the 16s rDNA of *Agrobacterium rhizogenes* indicate that based on monophyletic nature and phenotypic generic circumscription the genera *Agrobacterium, Allorhizobium* and *Rhizobium* are related and joined into one genus *Rhizobium* (Young et al. 2001).

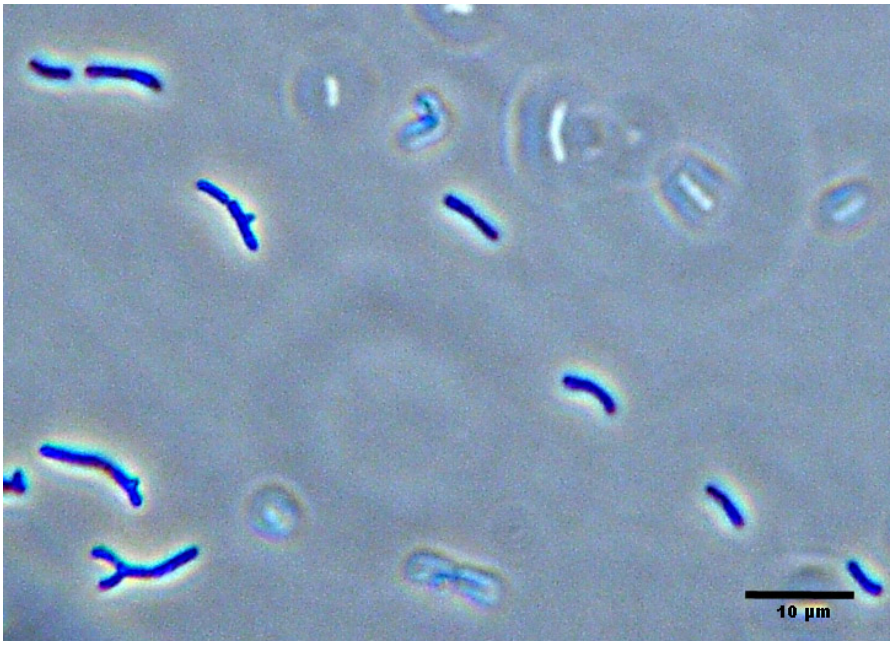

Figure 3: *Agrobacterium rhizogenes* (strain ATCC 15834) cultured in YMB medium for 2 days, dark field in light microscope

Agrobacterium rhizogenes is responsible for a neoplastic outgrowth of fine roots at the infection site, and infected plants show reduced vitality. These symptoms came to be known as the “hairy-root disease”. Transformed roots produce opines such as agropine, mannopine or cucumopine. Depending on the bacterial strain these metabolites are used as a carbon and nitrogen source for the bacteria. Strains of *Agrobacterium rhizogenes* are classified by the type of opines they produce, for instance the agropine, mannopine or cucumopine type. Experiments on comparative mapping and DNA hybridization point out that the DNA which is important for the hairy-root transformation is highly conserved. Since the 1980s, transformed roots induced by *Agrobacterium rhizogenes* are cultured *in vitro* because of their rapid growth without any requirement for exogenous phytohormones (Hamill and Lidgett 1997).

The virulent strains of *Agrobacterium rhizogenes* contain the Ri (root inducing) plasmids with different gene sequences (figure 4). The T-DNA (transferred DNA) is the DNA which is transferred from the bacterium to the plant cell. These segments, approximately 10-30 kbp in size, encode for the Ri conjugation, opine synthesis, catabolism, initiation, transfer and integration of the T-DNA itself. The borders of the T-DNA region are homologous and 25 bp in length.

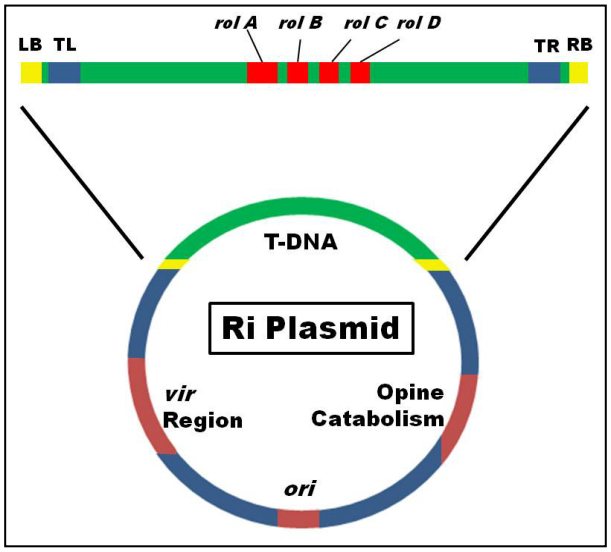

Figure 4: Schematic overview of a Ri plasmid of *Agrobacterium rhizogenes* (modified after Chandra 2012)

Plasmids can be divided in strains producing mannopine and cucumopine with single DNA and strains producing octopine and agropine with two T-DNAs. The two T-DNAs are classified in the TR-DNA (right DNA) and the TL-DNA (left DNA). The root-inducing genes (*rol A, rol B, rol C, rol D*) are found in the center of TL-DNA of the agropine-producing strains. A part of the TR-DNA are genes for the biosynthesis of auxins and the synthesis of mannopine and agropine. After the transfer of the TL-DNA and TR-DNA they are integrated in the genome of the plant cell. The TL-DNA is vital for the hairy-root induction (Chandra 2012).

Plant cell transformation

When plant cells are damaged, e.g. by an animal, they emit a simple phenolic substance, acetosyringone (figure 5, p. 8). Chemotactic effects attract *Agrobacterium rhizogenes*, which anchors to the wounded spot. The bacterium transforms the plant cell by integrating the T-DNA of its large Ri plasmid (root-inducing plasmid) into the genome of the infected plant. The vir region on the plasmid encodes for the T-DNA transfer which results in tumor-like hairy-root growth at the infected area. After integration of the new genes the onc genes, also located on the plasmid, support the tumor-like growth. A new hormonal balance in the plant cell induces proliferation of hairy roots at the wounded site (Guillon et al. 2006). Hairy roots can be induced on nearly every plant organ. As soon as gene expression occurs, the transformed plant cell starts to produce opines. As stated before, these opines are used as a carbon and nitrogen source by the bacteria and cannot be metabolized by the host plant (Dingermann and Zündorf 1999). *Agrobacterium rhizogenes*, closely related to *A. tumefaciens*, is used for genetic engineering by cloning gene sequences into the T-DNA of the Ri plasmid, which then is inserted into the host genome (Hamill and Lidgett 1997). Furthermore, genes that encode for auxin und cytokinin biosynthesis are also integrated into the plant genome (Guillon et al. 2006). The following figure 5 (page 9) illustrates the process of DNA transfer into the plant genome.

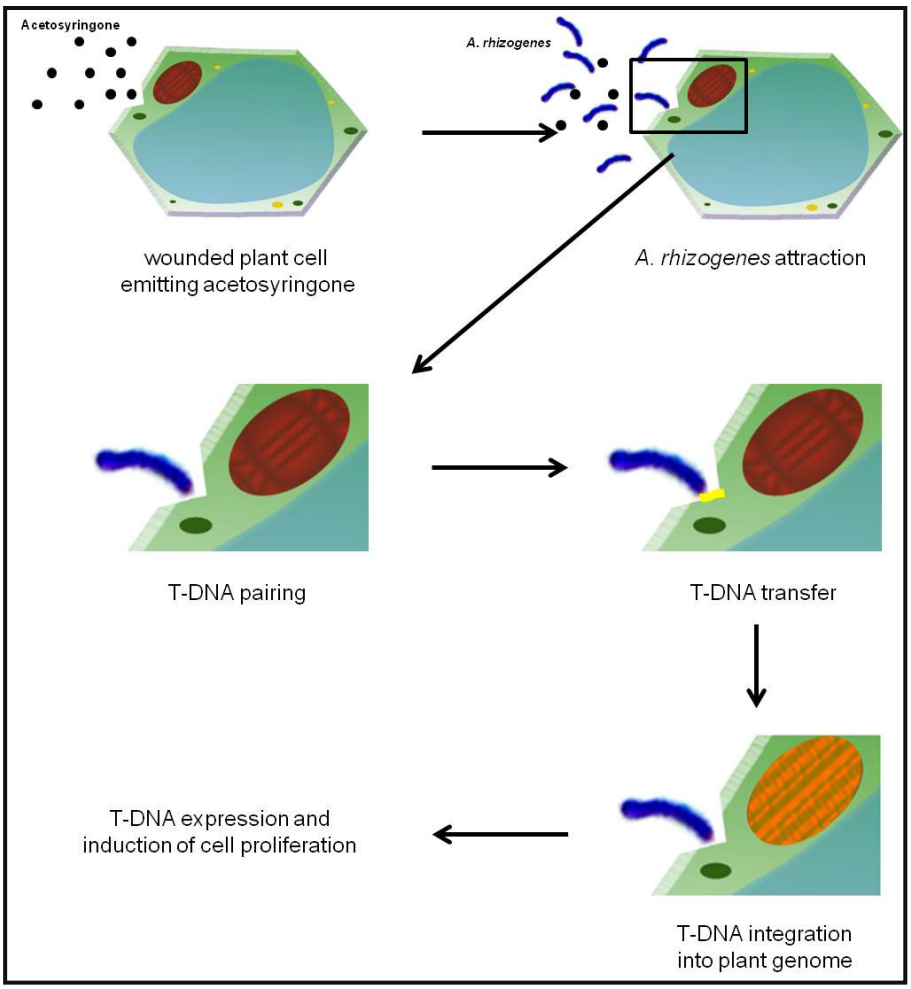

Figure 5: Procedure of the transfer of the *Agrobacterium rhizogenes* T-DNA into the plant genome

Secondary metabolites and hairy roots

In the 1930s, first studies on the *in vitro* cultivation of non-transformed roots were carried out. Roots of *Solanum lycopersicum* were cultured in a simple salt mixture containing yeast extract (White 1934). In 1942, tobacco was used for *in vitro* studies, which showed that nicotine was produced by these root cultures, but most of the nicotine was found in the medium (Dawson 1942). Despite the fact that several studies showed high levels of secondary metabolites of fast growing, non-transformed roots, in most reports growth regulators needed to be added to the established cultures with moderate growth. For this reason most of the further studies focused on disorganized cell suspensions (callus cultures) to obtain secondary metabolites. However, besides the fast growth rate of disorganized cell cultures many studies show

that they tend to be unstable and unreliable in regard to the production of secondary metabolites (Hamill and Lidgett 1997).

On the other hand hairy-root cultures established with *Agrobacterium rhizogenes* often show a rapid growth comparable to cell suspensions, but they are fully differentiated tissues which produce characteristic metabolites of the transformed plant. Yet, in contrast to cell-suspension cultures, hairy roots show a high chromosomal stability. Also, hairy roots tend to grow fast without any growth regulators added to the culture medium. It is possible that secondary metabolites which are produced in other tissues of the plant, e.g. leaves, are not synthesized in hairy roots. For example, studies on *Catharanthus roseus* revealed that vindoline and vinblastine were found in leaves of *in vitro* grown shoot cultures, but only very small amounts were present in hairy-root cultures of this plant (Parr et al. 1988). However, recent studies show that hairy roots are also capable of accumulating the same secondary metabolites produced in the aerial parts of the plant (Kim et al. 2002). *Lawsonia inermis* hairy roots cultured in darkness in MS medium are able to accumulate lawsone, usually formed in the upper parts of this plant (Bakkali et al. 1997). Likewise, artemisinin was known to be produced in the aerial parts of *Artemisia annua* (Wallaart et al. 1999), but studies show that hairy roots are able to produce artemisinin as well (Wang et al. 2001; Liu et al. 2002; Wang and Tan 2002). Hairy roots are proved to be genetically and biochemically stable and have the ability to synthesize natural compounds (Christey and Braun 2004; Georgiev et al. 2007; Srivastava and Srivastava 2007), as well as other compounds which are not found in non- transformed roots; for instance transformed roots of *Stellaria baicalensis* Georgi produce glucoside conjugates of flavonoids rather than the glucose conjugates of non-transformed roots (Nishikawa et al. 1999).

The biochemical and genetic stability of hairy roots also offers advantages compared to cell suspensions. It is reported that after a cultivation period of 20 weeks hairy roots of *Valeriana wallichii* DC induced with *A. rhizogenes* contain an amount of valepotriate which is 2 to 3.3 times higher than in non-transformed roots (Banerjee et al. 1998).

1.3. Aim of this study

This thesis addresses the possibility of establishing hairy roots in *Peucedanum ostruthium* by developing and testing a series of techniques and methods indicating how the use of three different wild-type strains of *Agrobacterium rhizogenes* (LBA 9402, TR 105, ATCC 15834) affect the formation of hairy roots. Given the evidence that different wild-type strains vary in their capability on hairy root induction and exhibit a different influence on the production of secondary metabolites, an examination of these factors seems essential (Ionkova and Fuss 2009).

The first aim deals with establishment of hairy roots. To answer this question, we begin by taking a closer look at the plant-wounding techniques: Generally, one prospect is to scratch petioles and leaves several times at the leaf veins with a surgical blade and to pipet bacterial suspension culture on the wounded region. Another procedure would be to dip a surgical blade into the bacterial suspension culture before scratching. Also, the practicability of cutting off leaves of plantlets and dipping the cut end of the petiole into the bacterial culture was to be investigated. Plants cultivated *in vitro* as well as such grown *in vivo* were to be compared. Subsequently, transformed hairy roots were to be grown in three different basal media, so that one could observe the effects that these media would have on root growth.

In the second part of this study, the effects of the bacterial strain used for transformation, and the effects of the culture medium on the roots' secondary metabolism, especially the coumarins in transformed roots, were to be examined. Hairy roots promise to be faster in growth, and to produce more biomass than non-transformed *in vitro* cultivated roots, still being closely bound to the production of secondary metabolites. Identification of the coumarins and furanocoumarins was to be achieved by HPLC-DAD analysis, and was to be compared with previous studies of non-transformed, rhizomes grown in nature.

Figure 6 on page 11 shows the main procedural steps to gain the results for this study.

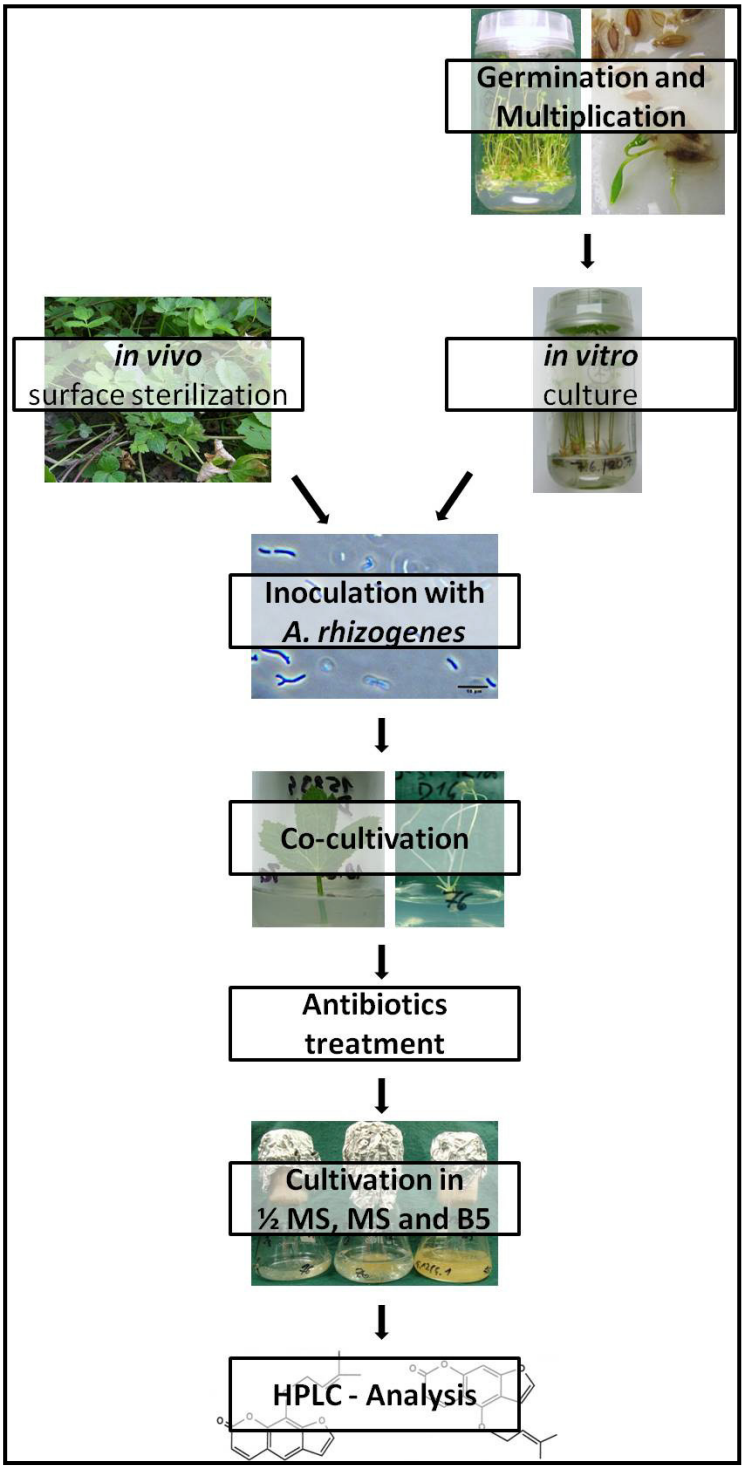

Figure 6: A schematic overview of the methods and main steps used in this diploma thesis (the term co-cultivation refers to the cultivation of two or more different organisms in one culture vessel)

2. Materials and Methods

2.1. Plant material

Shoot cultures were established within the context of the Austrian Science Fund (FWF) NFN-Project S10704-B037. Seeds were purchased from the company Jelitto Staudensamen GmbH, Schwarmstedt, Germany. A total of 720 seeds were surface-sterilized (see below), with 288 seeds pretreated by soaking them for 24 hours in an aqueous solution of 500 ppm gibberellic acid. Afterwards they were washed and dried at 35°C in a drying chamber before the germination started. Seed germination was performed in 58 mL test tubes containing 13 mL of ½ MS medium (refer to chapter 2.3, page 16) without any growth regulators. Half of the seeds were germinated at 25±1°C in a 16-hours photo period with a light regime of 50 $\mu M \cdot m^{-2} \cdot s^{-1}$ (SYLVANIA GRO-LUX® fluorescent tubes) and the other half was pretreated for four weeks at 5±1°C in darkness before transferring them to the above mentioned conditions. Shoot multiplication was maintained on ½ MS medium containing 0.5 µM benzylaminopurine. After the multiplication stage the shoot cultures were kept on ½ MS medium without growth regulators, under the conditions described above.
In vivo plants had been collected and identified by J. Saukel (Department of Pharmacognosy, Vienna) and were readily available in the gardens of the department.

Surface sterilization

All leaves taken from the open ground needed to be surface-sterilized before inoculation. The leaves were first washed thoroughly under running tap water. Subsequently, they were submerged in 10% ethanol for 5 minutes and then treated with aqueous solutions of sodium hypochlorite (W. Neuber's Enkel GmbH) for 20 minutes. Two different concentrations of sodium

hypochlorite (1.7% and 2.1% active chlorine, respectively) were tested. The explants were then washed four times with autoclaved water.
The surface sterilization of seeds was performed basically in the same way but with some modifications: The samples were submerged in 30% ethanol for 10 minutes and the concentration of the aqueous solution of sodium hypochlorite was adjusted to 4.25 active chlorine, with a treatment duration of 30 minutes.

Types of explants

Different types of explants were used in successive experiments. On the one hand, leaves were excised from *in vivo* or *in vitro* grown plants, trimmed to a size of approximately 2 cm^2 (*in vitro*) to 8 cm^2 (*in vivo* plants), and the leaf veins slightly scratched with a surgical blade. On the other hand, whole *in vitro* cultivated plantlets were used, with petioles scratched. Finally, the leaves of *in vitro* grown plants were removed at approximately half the length of the petiole and the remaining “cut” plant was used for infection.

2.2. *Agrobacterium rhizogenes*

In the present study three different wild-type strains were used: LBA 9402, TR 105, and ATCC 15834. We are grateful to Prof. Dr. Maike Petersen, Institute of Pharmaceutical Biology and Biotechnology, Philips University of Marburg, for providing the LBA 9402 and TR 105 strains. The strain ATCC 15834 was purchased from LGC Standards, Germany.
The bacteria were grown in 100 mL Erlenmeyer flasks with 20 mL liquid YMB medium (see chapter 2.3. on page 16). All samples were incubated on a rotary shaker for 2 days with 70 rpm at a room temperature of 25±1°C and 70% relative humidity.
For long term storage the bacteria were cultured in petri dishes on YMB medium at 4°C.

2.2.1. Hairy-root induction

Leaves from *in vivo* grown and *in vitro* grown cultures were scratched several times at the leaf veins with a surgical blade that had been dipped into the bacterial suspension culture. Petioles from *in vitro* cultures were scratched the same way.

Alternatively, petioles and leaf veins of *in vitro* grown plantlets were scratched, but now the bacterial suspension culture was pipetted on the wounded region. Also, leaves of *in vitro* grown plantlets were cut off, and the cut end of the petiole dipped into the bacterial culture. Figure 7 illustrates the different wounding strategies. All explants were then further co-cultivated on ½ MS medium (the term "co-cultivation" refers to the cultivation of two or more different organisms in one culture vessel).

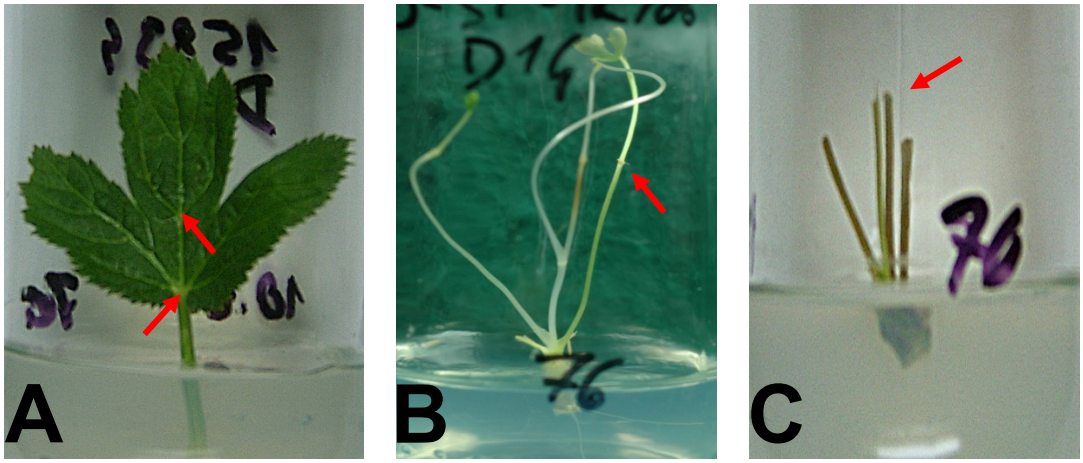

Figure 7: *Peucedanum ostruthium* wounding areas for the co-cultivation with different *Agrobacterium rhizogenes* strains; (A) explants established from *in vivo* samples – scratched leaf veins; (B) explants established from *in vitro* samples – scratched petioles; (C) explants established from *in vitro* explants – leaves cut off

2.2.2. Rhizogenesis

After the bacterial inoculation, the samples were kept at 25±1°C, with a shelf temperature of 23±1°C and a relative humidity of 70%. One group of samples was kept under a 16-hours photo period, the other one was kept in complete

darkness – both groups were stored for 3 to 4 weeks in baby food jars with 40 mL of solid ½ MS medium (see chapter 2.3, page 16).
When hairy roots had formed, they were cut off from the primary explants and inoculated into 20 mL of each of three basal media in 100 mL Erlenmeyer flasks and treated three times with 0.5 g/L of the antibiotic Cefotaxim-Na (Claforan®). Each treatment lasted 3 weeks. At the end of the subculture a sample of the liquid medium was inoculated on YMB medium for 2 days to monitor the presence of bacteria.

2.2.3. Hairy-root culture

Seven fast-growing hairy-root clones were cultivated for six weeks after the antibiotics treatment and labeled in the following way:
"L", "T", or "A" is the code for the bacterial strains LBA 9402, TR 105, and ATCC 15834, respectively, which were used for the induction of hairy roots.
"HM", "MS", or "B5" are codes for the nutrient medium (½ MS, MS and B5 medium, respectively).

Upon availability, hairy-root clones were inoculated into 50 mL of each of the three basal nutrient media, utilizing 250 mL Erlenmeyer flasks. The hairy roots were sub-cultivated every 3 to 4 weeks and grown in darkness on a rotary shaker at 70 rpm, at a room temperature of 25±1°C and a relative room humidity of 70%. After each cultivation cycle the fresh weight was measured. When three cultivation cycles had been completed, the hairy roots were dried for 2 days at room temperature, and the dry weight was determined.

2.3. Culture media

Plant-culture media

MS and the modified ½ MS media were based on the formulation of Murashige and Skoog (1962), and the B5 medium on the formulation of Gamborg et al. (1968). All chemicals used in this study were of standard quality except of the sucrose which was of household grade. For easier

handling the stock solutions were prepared as follows: Each macronutrient (100-fold stock) was weighed on a micro scale and dissolved in 1 L of distilled water and stored in the refrigerator at 4°C. For the $FeSO_4 \cdot 7H_2O$ solution (100-fold stock), Na_2-EDTA was dissolved first before the $FeSO_4 \cdot 7H_2O$ was added. Stock solutions of vitamins (200-fold) and micronutrients (1000-fold) were prepared in the same way and filled up to a final volume of 500 mL.

For media preparation, myo-inositol (100 mg/L) was suspended in distilled water and the amount of stock solution for each medium (10 mL/L macronutrients, 5 mL/L vitamins and 1 mL/L micronutrients) was added. ½ MS medium was prepared with 5 mL/L macronutrients. The sucrose concentration was 3% in all three media, and the pH value was adjusted to 5.7±0.1 with aqueous KOH.

Before autoclaving, the medium was filled into 100 mL Erlenmeyer flasks (20 mL medium) or 250 mL Erlenmeyer flasks (50 mL medium). The flasks were closed with cellulose plugs and autoclaved for 20 min at a pressure of 1 bar and a temperature of 121°C. All media were stored in darkness at 5°C for further use.

For solid media, 3 g/L Gelrite® was added and 40 mL of medium were filled into 250 mL baby food jars. For the multiplication of shoot cultures the growth regulator benzylaminopurine was added to the medium before autoclaving at a concentration of 0.5 µM. After closure with Magenta B-caps the jars were autoclaved as described above.

13 mL of germination medium (solid ½ MS) was filled into 58 mL test tubes and closed with Magenta 2-way caps and autoclaved like the other media.

A. rhizogenes culture media

YMB medium (Wright et al. 1930) was used to cultivate the bacteria. The pH value was adjusted to 7.2±0.1 with aqueous KOH and autoclaved in 100 mL Erlenmeyer flasks as described above.

For the solid YMB medium, 8 g/L agar (MERCK) was added before autoclaving. After autoclaving, 30 mL of the medium were dispensed into petri dishes (60 mm) under sterile conditions.

2.4. Identification and quantification of hairy roots by HPLC-DAD

For the coumarin analysis, 0.1 g of powdered hairy roots was extracted with 4 mL of dichloromethane using reflux extraction for 15 min at 40°C. Subsequently, the obtained solution was filtered, evaporated to dryness, and re-dissolved in 0.5 mL of methanol for HPLC analysis.

The HPLC instruments used for this analysis were made by Shimadzu (Kyoto, Japan). A CBM-20A system controller, a DGU-20A5 membrane degasser, an LC-20AD solvent delivery unit, an SIL-20AC HT autosampler, a CTO-20AC column oven, and an SPD-M20A photodiode array detector were used for quantification (Vogl et al. 2011).

Data analysis was performed using the chromatography software LCsolution 1.2 (Shimadzu).

Chromatographic separation was achieved on a 150 mm 2.1 mm i.d., 3 µm, Acclaim 120 C18 reversed-phase column, with a 10 mm 2.1 mm i.d., 5 µm, Acclaim 120 C18 guard column from Dionex (Germering, Germany), at 38°C and a flow rate of 0.5 mL/min. Water (modified with 0.01% acetic acid) and MeCN were used as mobile phases A and B, respectively. Gradient elution was performed as follows:

25-37% of B in 6 min, 37-45% of B in 8 min, 45-65% of B in 10 min, 65-95% of B in 1 min, and isocratic at 95% of B for 5 min. The injection volume was 20 µL for each sample (Vogl et al. 2011).

To identify the main coumarins, chromatograms and UV spectra were compared at 310 nm to the seven major coumarins identified by Vogl et al. (2011) via HPLC-DAD. Imperatorin with a purity of 99% was purchased from Herboreal Ltd. (Edinburgh, UK) and was chosen as an internal standard.

3. Results

3.1. Establishment of shoot cultures

Before the establishment of *in vitro* cultures was started, the seed germination ability had been tested by inoculating seeds in petri dishes on wet filter paper disks. As illustrated in figure 8, the germination rate was rather poor, therefore we also tested a pretreatment of the seeds with gibberellic acid (500 ppm aqueous solution, soaking for 24 hours). Additionally, we also subjected the seed cultures to a four-week cold treatment at 5°C before the transfer to the standard 25°C condition. It is known that a cold pretreatment can reduce the period of dormancy.

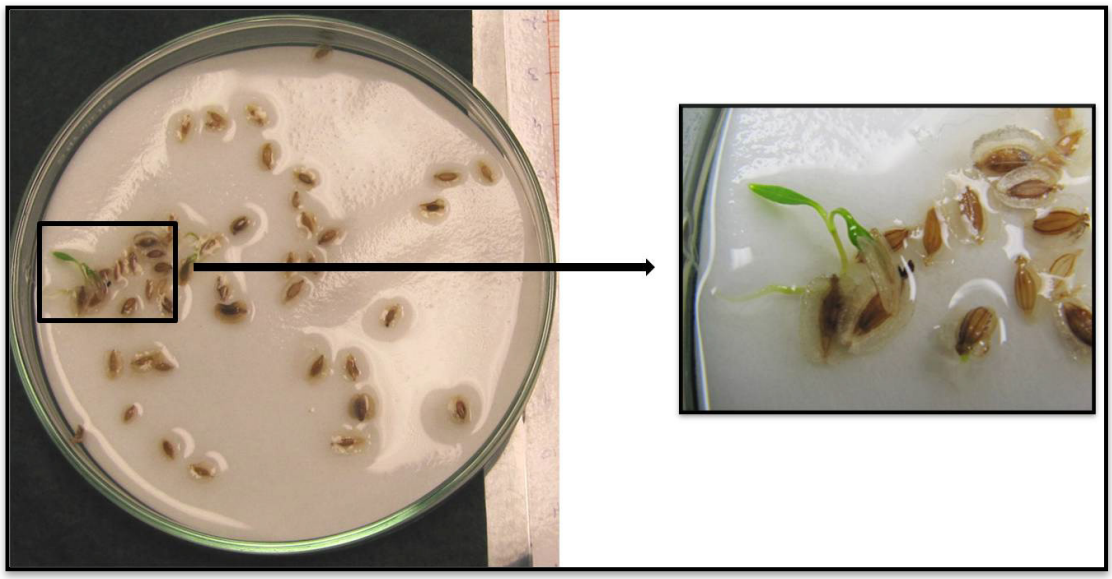

Figure 8: Testing the germination ability of *Peucedanum ostruthium* seeds. left: petri dish with wet filter paper and seeds, right: seedling of masterwort

Out of the 720 seeds which were singly inoculated for germination (see table 1 on page 20), 334 cultures turned out to be contaminated. In 329 cultures which could be established in aseptical culture, just 57 seeds germinated in total. The four-week pretreatment at a temperature of 5±1°C had a noticeable

influence on the germination: 43 plantlets developed under this condition, while only 14 seeds germinated without the cold pretreatment. Both the pre-treatment with gibberellic acid and the cold-temperature treatment turned out to positively influence the germination of the *Peucedanum ostruthium* seed batch which was used in the present study.

Table 1: Germination of *Peucedanum ostruthium* seeds established at temperatures of 5°C and 25°C, with and without gibberellic acid pretreatment

pretreatment	T (°C)	seeds	contaminated	cultures established	seedlings	%*
-	25	216	105	111	11	10
-	5	216	92	124	24	19
GA3	25	144	78	66	3	5
GA3	5	144	59	86	19	22
* germination rate						

After six to eight weeks several shoots formed on the multiplication medium. The multiplication was performed on solid ½ MS medium containing 0.5 µM benzylaminopurine (figure 9 on page 21, A). The shoots were separated and 5 to 6 shoots were inoculated for 6 weeks on solid ½ MS medium. Clone POS9 of the established shoot cultures showed the best performance in propagation. For this reason this clone was chosen for further co-cultivation with *Agrobacterium rhizogenes* (figure 9 on page 21, B).

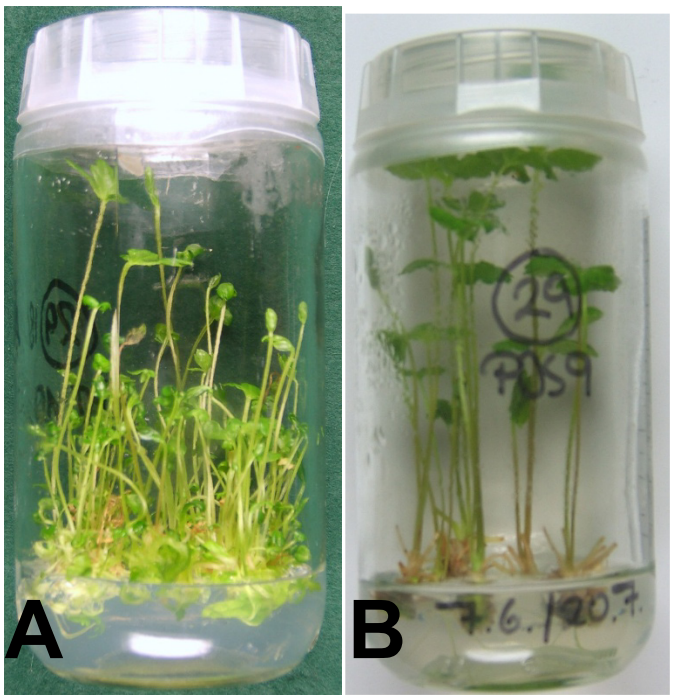

Figure 9: Shoot cultures of *Peucedanum ostruthium* (A) Multiplication on ½ MS medium containing 0.5 µM benzylaminopurine, (B) Culture for inoculation with *Agrobacterium rhizogenes* on ½ MS medium after multiplication

To investigate whether *Agrobacterium rhizogenes* is able to induce hairy-root formation in *Peucedanum ostruthium*, explants of *in vivo* and *in vitro* plants were wounded and incubated with the bacterial culture. In case the T-DNA transfer of the *Agrobacterium* to the host plant was successful, hairy roots emerged at the wounded area after 3 to 4 weeks, as shown in figure 10 on page 22.

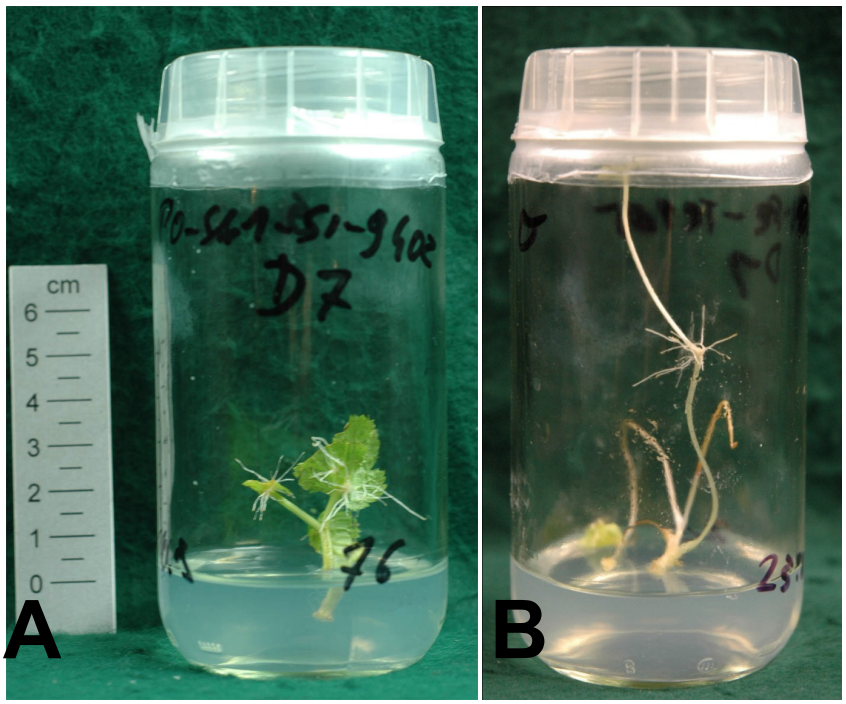

Figure 10: *Agrobacterium rhizogenes*-mediated hairy-root induction in *Peucedanum ostruthium* after 3 to 4 weeks on ½ MS medium (A) *in vivo* explant with hairy-root formation at scratched leaf veins; (B) *in vitro* explant with hairy-root formation at scratched petiole

3.2. Plant-wounding techniques

Generally, a number of different infection techniques have been described for the establishment of hairy-root cultures. Therefore we tested different approaches in order to achieve successful infection, and further hairy-root formation, on a selected clone of *P. ostruthium*, and also on leaf explants taken from plants growing naturally, i.e. *in vivo*.

In vitro grown shoot cultures that were just decapitated (”cut” plantlets: the leaf blades were cut off), and dipped into the bacterial suspension culture with the cut part of the petiole, did not form any hairy roots at all. Thus, our experiments indicate that this infection method is not suited for the establishment of hairy roots in our given *P. ostruthium* clone, and with the utilized *Agrobacterium* strains.

In contrast, six samples of scratched leaves petioles that had bacterial suspension culture pipetted on the wounded region had produced hairy roots. In these explants *Agrobacterium rhizogenes* strain LBA 9402 did not show any hairy-root formation with this technique. Both TR 105 and ATCC 15834 induced hairy roots at the wounded area three times each (Figure 11).

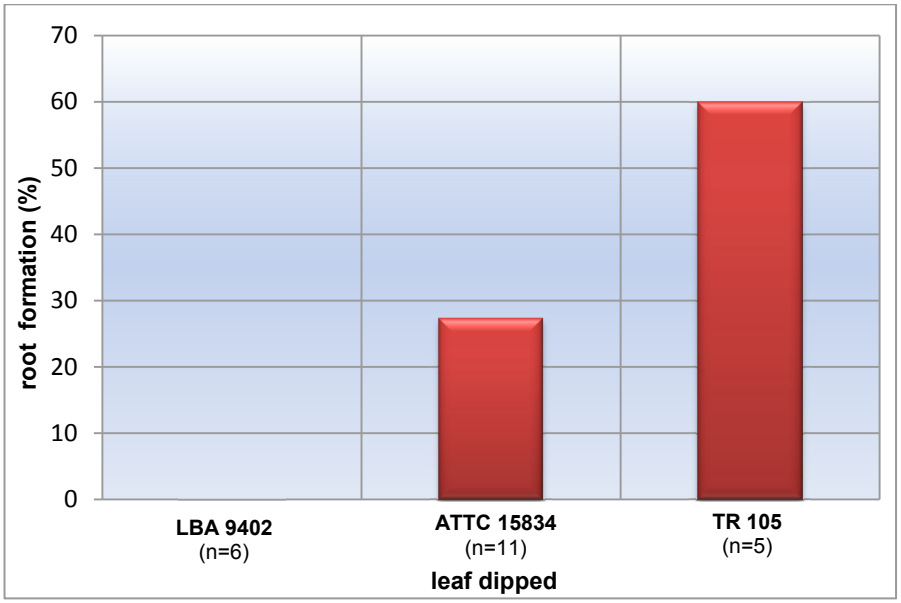

Figure 11: Hairy-root induction in *P. ostruthium* of scratched leaf veins that had been pipetted on with *Agrobacterium rhizogenes* strain LBA 9402, ATCC 15834 and TR 105

Moreover, five samples of petioles that had bacterial suspension culture pipetted on their scratched side had produced hairy roots. Hairy roots established with *Agrobacterium rhizogenes* strain LBA 9402 and ATCC 15834 each showed hairy-root formation in one explant only. TR 105 formed hairy roots three times and was the strain performing best with this technique (Figure 12, p. 24).

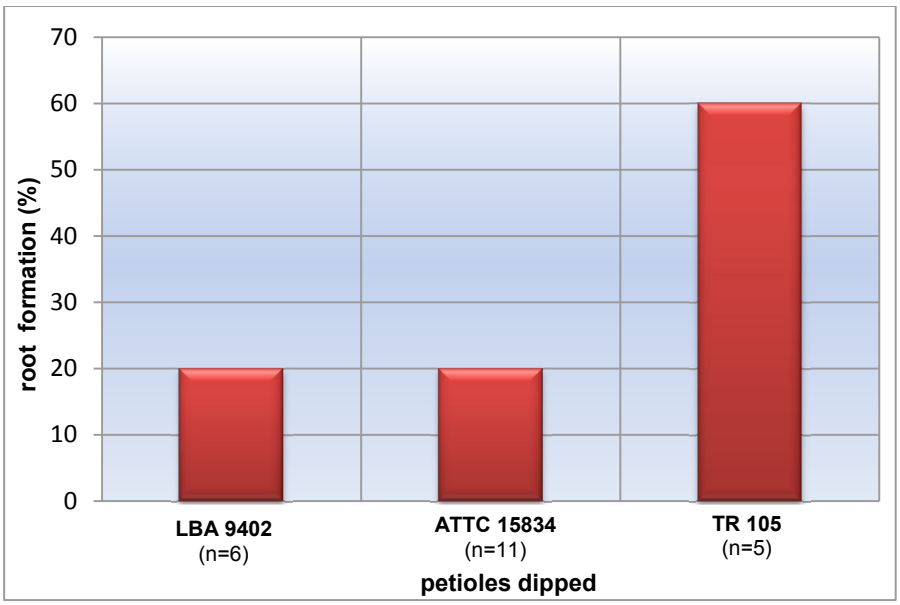

Figure 12: Hairy-root induction in *P. ostruthium* of scratched petioles, pipetted-on with *Agrobacterium rhizogenes* strain LBA 9402, ATCC 15834 and TR 105

Although the pipetting process was carried out very carefully, in some cases bacterial suspension dripped onto the nutrient medium. As a consequence, bacterial growth on the medium eventually resulted in death of the explants.

For all *Agrobacterium rhizogenes* strains used in this study the best way to obtain hairy roots was to scratch the petiole. The surgical blade was dipped into the bacterial suspension culture before scratching, thus no excess bacterial suspension could drip on the nutrient medium. Strain LBA 9402 induced 9 hairy roots, ATCC 15834 16 and TR 105 25 hairy roots (Figure 13, p. 25). The root-formation rate for strain TR 105 was always the highest except in this case. Strain ATTC 15834 (55%) showed a little higher transformation rate than TR 105 (54%) with this technique. The same is true for strain that LBA 9402 (43%) showed the highest transformation rate when treated with this technique in contrast to all other techniques used on this strain.

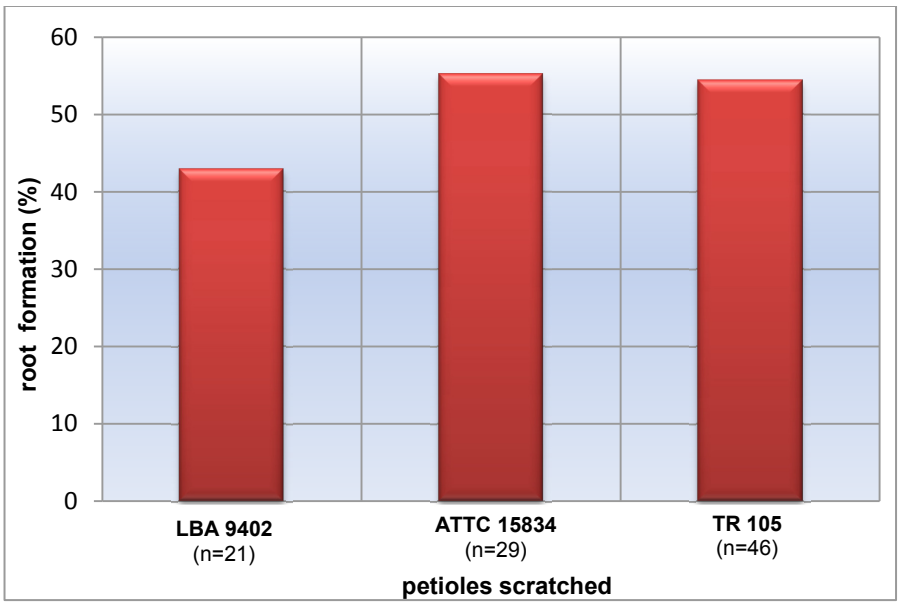

Figure 13: Hairy-root induction in *P. ostruthium* of scratched petioles with a surgical blade with *Agrobacterium rhizogenes* strain LBA 9402, ATCC 15834 and TR 105

The results obtained from all different techniques and all *A. rhizogenes* strains with *in vitro* grown plantlets are shown in figure 14, page 26. Furthermore all plant wounding techniques are summarized in table 2. Explants which were scratched and which the bacterial suspension culture was pipetted on were similar in regard of the transformation rate of 18% and 23%. It is shown that scratching the petioles with a surgical blade which was dipped into the bacterial suspension culture before resulted in the highest transformation rates of around 50%. This technique was the best suited for our hairy-root induction in *Peucedanum ostruthium*. Through all transformation techniques, except the “cut”-plantlets technique used in this study, the strain TR 105 showed the best results. Root-formation frequency is given as the number of explants forming hairy roots, divided by the total number of explants, and multiplied by 100.

Table 2: Results of plant-wounding techniques with all three different *Agrobacterium rhizogenes* strains

strain	leaf dipped (%)	petiole dipped (%)	petiole scratched (%)	"cut" plantlets (%)
LBA 9402	0	20	43	0
ATCC 15834	27	20	55	0
TR 105	60	60	54	0

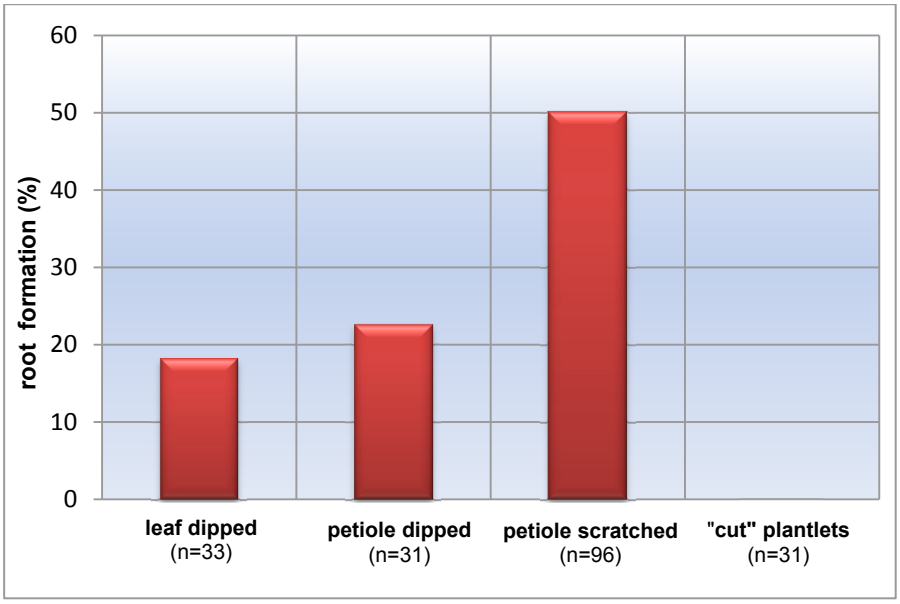

Figure 14: Influence of wounding techniques on hairy-root induction in *P. ostruthium* infected with different *Agrobacterium rhizogenes* strains

3.2.1. Co-cultivation of *A. rhizogenes* and *P. ostruthium*

Within our studies a total of 381 samples of *Peucedanum ostruthium* (191 *in vitro*-explants and 190 *in vivo*-explants) were inoculated with *A. rhizogenes*, applying the different techniques previously described. Out of this total number, 174 samples did not show any formation of hairy roots. In 86 cultures the explants died during the cultivation, and further 60 cultures turned out to be contaminated with *Agrobacterium* suspension culture that had dripped on the medium, or were unusable because of an unsuccessful surface sterilization.

Out of all samples, 61 showed hairy-root formation at the wounded area.

In vivo explants which were only scratched at the leaf veins showed a very low hairy-root formation rate in contrast to *in vitro* explants. Of the 190 *in vivo* explants only 3 formed hairy roots (Figure 15, p. 27). In that case surface sterilization may have failed to render the explants sterile. Because of the low transformation rate of *in vivo* explants we focused on further experiments on explants taken from *in vitro*.

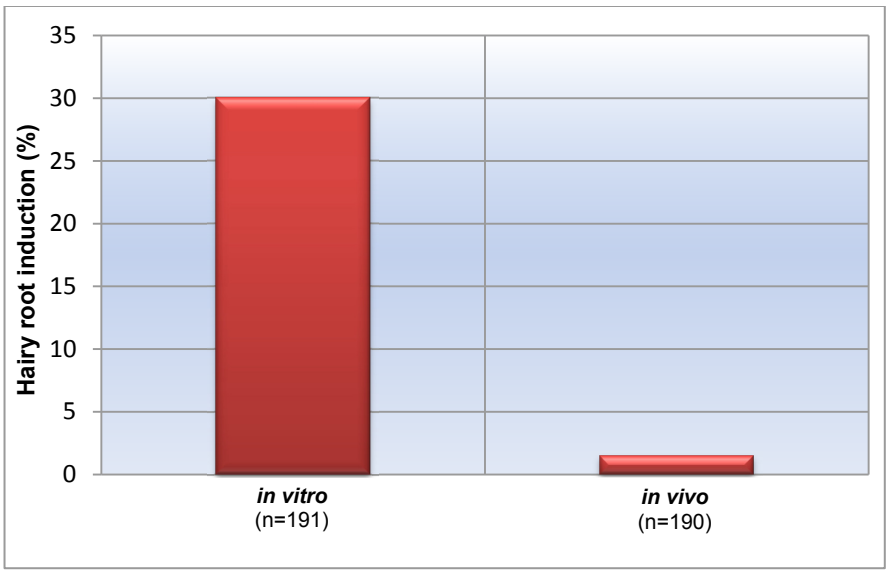

Figure 15: Hairy-root induction in *P. ostruthium* performed form explants taken from *in vitro* and *in vivo* plants and infected with different *Agrobacterium rhizogenes* strains

Peucedanum ostruthium showed no hairy-root formation under light conditions, as can be seen in figure 16. Neither the plant-wounding technique nor the bacterial strains used for this experiment had any influence on forming hairy roots under this condition. For this reason all further studies were carried out in complete darkness.

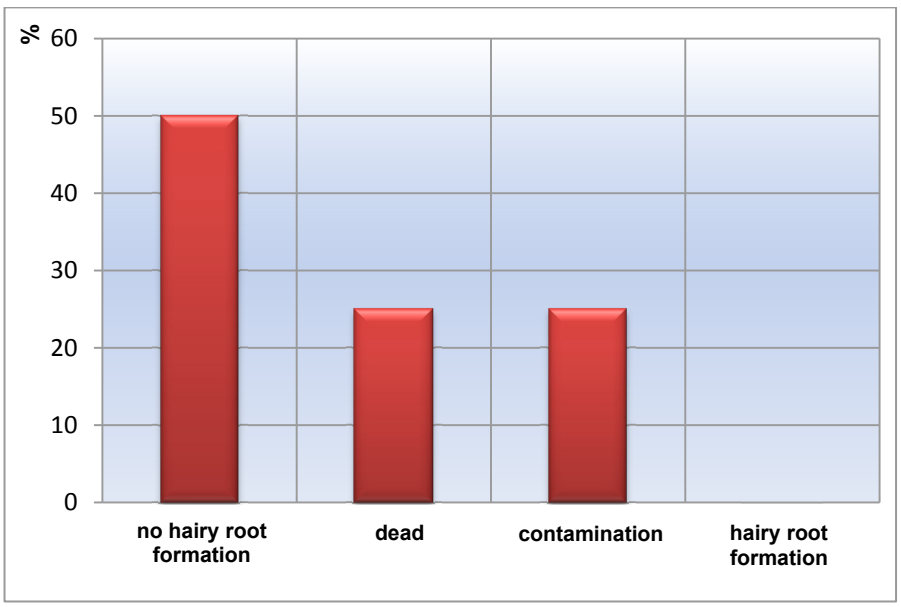

Figure 16: Results of co-cultivation in *Peucedanum ostruthium* infected with different *Agrobacterium rhizogenes* strains under light conditions (n=136)

After infection, 245 samples were cultivated in the dark. Out of these, 106 samples did not form hairy roots, while 51 explants died during the process and further 27 cultures became contaminated. On 61 explants (25%) hairy root-formation could be observed.

64 cultures were infected with *Agrobacterium* strain LBA 9402 (Figure 17), out of these explants 19% showed no hairy-root formation at all, 10% died during the cultivation and 6% were contaminated. Hairy roots were formed in 7% of the samples, which is the lowest transformation rate of all strains used in this thesis.

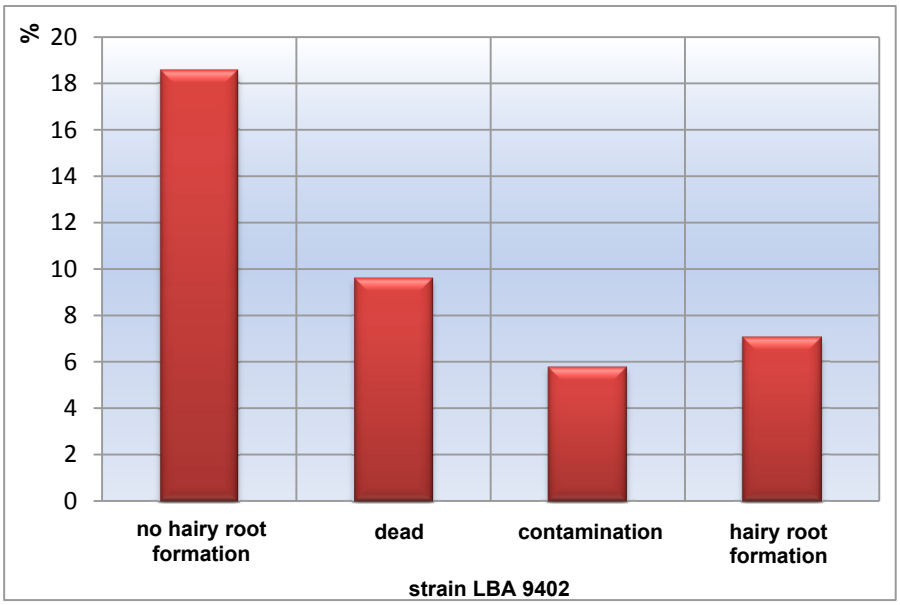

Figure 17: Results of co-cultivation in *Peucedanum ostruthium* infected with *Agrobacterium rhizogenes* strain LBA 9402 (n=64)

Figure 18 on page 29 shows the results of the infections done with strain ATCC 15834. No hairy-root formation was perceived for 34%, 21% came out to be dead and 9% were contaminated. 19% formed hairy roots, the second highest value of all three *Agrobacterium* strains.

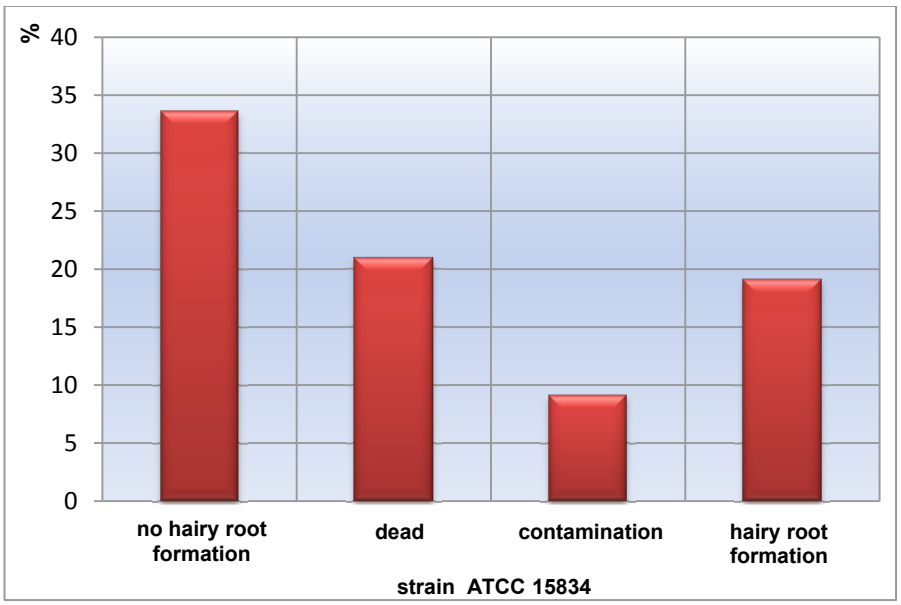

Figure 18: Results of co-cultivation in *Peucedanum ostruthium* infected with *Agrobacterium rhizogenes* strain ATCC 15834 (n=91)

In figure 19 the outcome of the co-cultivation of strain TR 105 and *Peucedanum ostruthium* is shown. This strain showed the highest transformation rate of 26% over all of the strains used in this study. 36% of 90 explants showed no hairy-root formation, 12% died during the process and 7% came out to be contaminated.

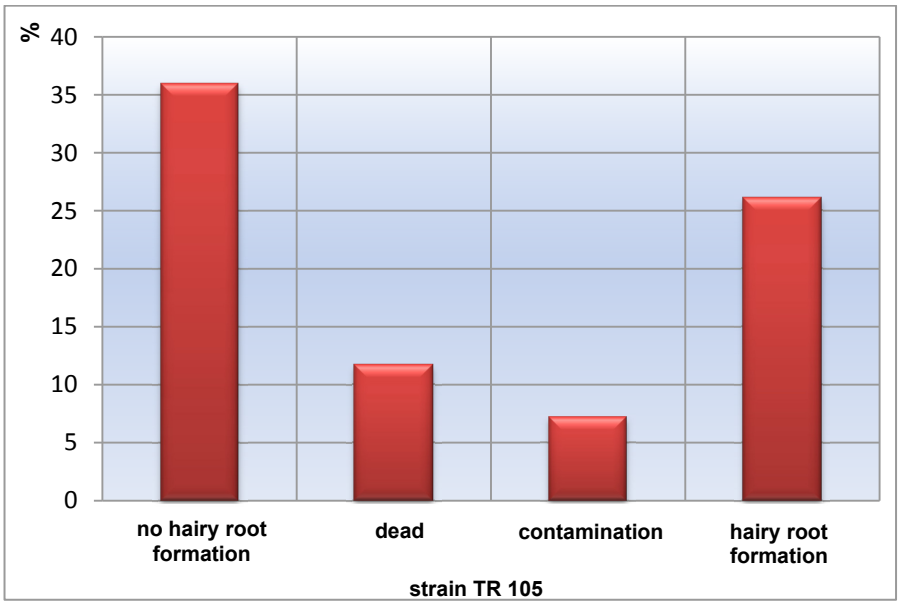

Figure 19: Results of co-cultivation in *Peucedanum ostruthium* infected with *Agrobacterium rhizogenes* strain TR 105 (n=90)

3.2.2. Virulence of *A. rhizogenes* strain

The three wild-type strains of *A. rhizogenes* exhibited a varying ability to induce hairy-root formation: strain TR 105 induced hairy-root formation in 29 samples, strain LBA 9402 on 11 explants, and strain ATCC 15834 in 21 cultures (Figure 20). Strain TR 105 showed the highest virulence for hairy-root induction, followed by strain ATCC 15834. The hairy-root induction frequency as detailed in figure 20 was calculated as the number of explants infected with a bacterial strain of *Agrobacterium rhizogenes*, divided by the total number of explants infected with this strain, and multiplied by 100.

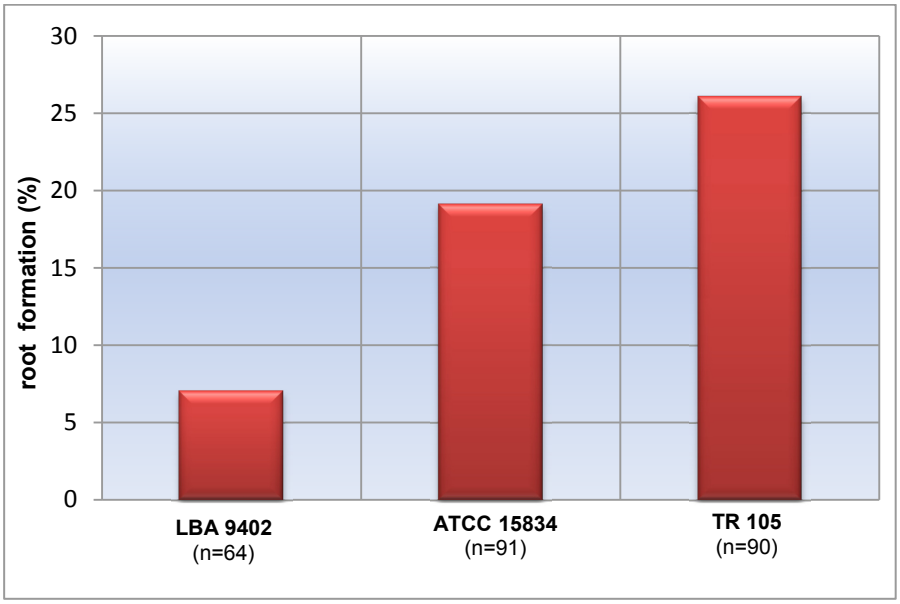

Figure 20: Frequency of hairy-root induction in *Peucedanum ostruthium* infected with different *Agrobacterium rhizogenes* strains cultivated in darkness

3.3. Influence of basal medium on hairy-root growth

It became evident that the choice of the basal medium had an influence on the growth rate, which also depended on the specific hairy-root clone. Figure 21 and figure 22 (page 31) illustrate the biomass increase of samples T22HM (½ MS medium), T22MS (MS medium) and T22B5 (B5 medium) infected with *A. rhizogenes* strain TR 105 after 6 weeks and 9 weeks of cultivation, respectively, depending on the basal media.

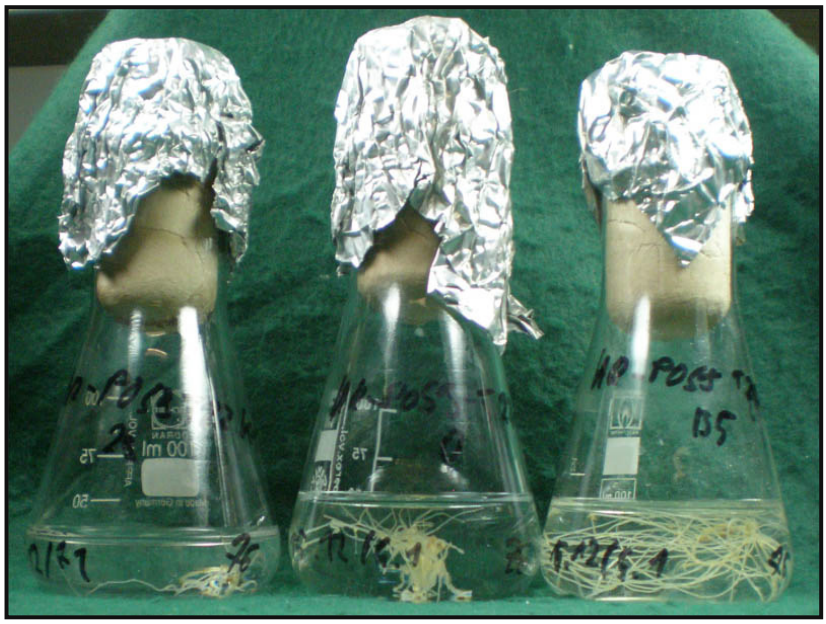

Figure 21: Hairy-root culture of *Peucedanum ostruthium* (*Agrobacterium rhizogenes* strain TR 105) after 6 weeks of cultivation in media ½ MS (left), MS (middle) and B5 (right)

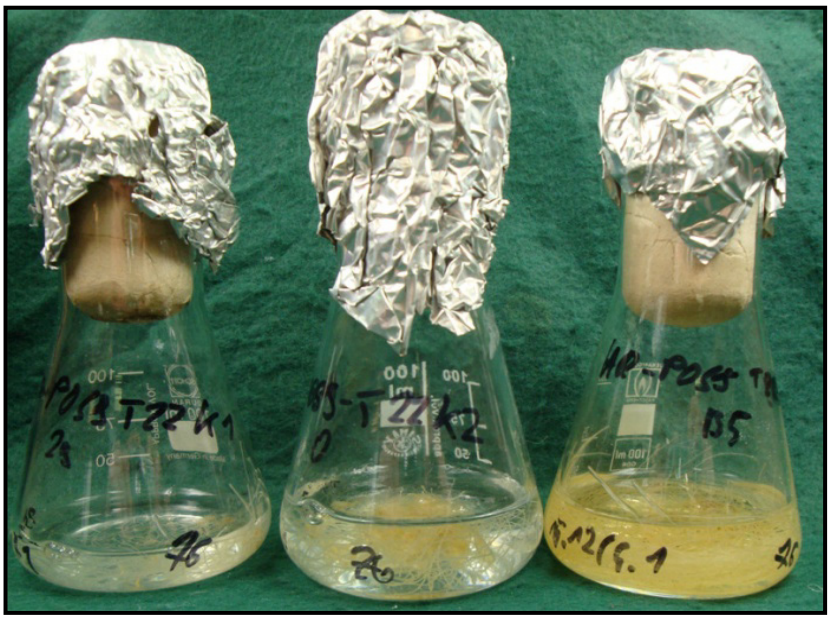

Figure 22: Hairy-root culture of *Peucedanum ostruthium* (*Agrobacterium rhizogenes* strain TR 105) after 9 weeks of cultivation in media ½ MS (left), MS (middle) and B5 (right)

The ½ MS nutrient medium in most cases resulted in moderate growth rates, except for samples T52 and T54, for which this medium seemed to be the most suitable.

The B5 nutrient medium turned out to be the most performant medium for hairy-root cultivation in this study with *Peucedanum ostruthium*. Nearly every sample showed a noticeable increase in biomass, from 1.5- up to 9-fold of the initial fresh weight.

Hairy roots established with *A. rhizogenes* strain LBA 9402 (sample L8) showed the least increase in growth, followed by sample A11 (strain ATCC 15834). All other hairy roots were established with strain TR 105 (see table 3 for details).

Table 3: Influence of culture media on hairy-root culture of *Peucedanum ostruthium,* established with different *Agrobacterium rhizogenes* strains, and cultivated in ½ MS, MS and B5 medium for 3 weeks

sample	flask	medium	start weight (g)	end weight (g)	increase (%)
T53 (strain TR 105)	HM	½ MS	0.29	0.372	28
	MS	MS	0.46	1.05	128
	B5	B5	0.343	0.706	106
A11 (strain ATCC 5834)	HM	½ MS	0.11	0.141	28
	MS	MS	0.025	0.035	40
	B5	B5	0.045	0.083	84
T51 (strain TR 105)	HM	½ MS	0.12	0.19	58
	MS	MS	0.054	0.108	100
	B5	B5	0.164	1.634	896
T22 (strain TR 105)	HM	½ MS	0.324	1.66	412
	MS	MS	1.525	7.326	380
	B5	B5	1.89	14.452	665
T54 (strain TR 105)	HM	½ MS	0.059	0.11	86
	MS	MS	0.09	0.188	109
	B5	B5	0.151	0.254	68
T52 (strain TR 105)	HM	½ MS	0.408	0.694	70
	MS	MS	0.073	0.112	53
	B5	B5	0.26	0.4	54

Table 3: continued:

sample	flask	medium	start weight (g)	end weight (g)	increase (%)
L8 (strain LBA 9402)	HM	½ MS	0.271	0.713	163
	MS	MS	0.673	1.425	112
	B5	B5	0.731	2.48	239

Hairy roots fresh and dry weight for HPLC analysis

All samples showed the same ratio (approximately 10:1) of fresh to dry weight and were subsequently subjected to HPLC analysis (table 4).

Table 4: Fresh and dry weight of hairy-root clones of *P. ostruthium* for HPLC analysis

sample name	flask	medium	fresh weight (g)	dry weight (g)
T22B5 (strain TR 105)	A	B5	2.061	0.249
	B	B5	4.183	0.396
	C	B5	3.364	0.323
	D	B5	1.662	0.173
T22HM (strain TR 105)	B	½ MS	0.765	0.060
	C	½ MS	0.562	0.047
T22MS (strain TR 105)	A	MS	2.025	0.188
	B	MS	1.926	0.154
	C	MS	2.755	0.230
T55B5 (strain TR 105)	I	B5	1.140	0.128
	J	B5	1.113	0.127
	K	B5	1.464	0.155
	L	B5	0.971	0.112
T57B5 (strain TR 105)	A	B5	2.023	0.228
T53B5 (strain TR 105)	D	B5	1.264	0.151
L8B5 (strain LBA 9402)	A	B5	1.732	0.186
L8MS (strain LBA 9402)	A	MS	1.013	0.115

3.4. HPLC Analysis

Four coumarins (oxipeucedanin, imperatorin, isoimperatorin and ostruthin) were identified by comparing their UV spectra at 310 nm and the retention times to those of the major coumarins identified in a previous study (Vogl et al. 2011). Figure 23 illustrates the main coumarins found in wild collection of *Peucedanum ostruthium* rhizome. The samples were collected in the Weisspriachtal in Salzburg. Ostruthol, osthol and peucenin could not be detected in the hairy-root lines established in this study.

On the one hand some coumarins and furanocoumarins were not detectable in our samples, but on the other hand unknown substances occurred in nearly all samples.

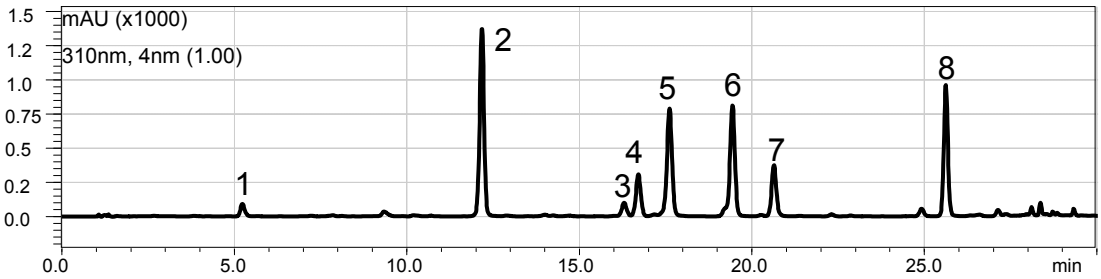

Figure 23: HPLC chromatogram (Vogl et al. 2011) of *P. ostruthium* rhizome from a wild collection in the Weisspriachtal, Salzburg. Peak numbers refer to oxypeucedanin hydrate (1), oxypeucedanin (2), peucenin (3), ostruthol (4), imperatorin (5), osthol (6), isoimperatorin (7) and ostruthin (8)

At first, the analyses of the dichloromethane extracts revealed that the concentration of the detectable coumarins (Table 5, p. 35) was distinctly lower than in wildly grown *Peucedanum ostruthium*. The sample T22B5 contained all four identified coumarins. In T22HM, no coumarins at all could be detected – most likely the concentrations were too low to obtain a clear result.

Imperatorin and isoimperatorin were found in 6 of all 8 analyzed samples.

No other known coumarins were found in the hairy roots of *Peucedanum ostruthium* used in this study, although some peaks showed a coumarin-like UV spectrum. These peaks did occur in various samples, but have not been further investigated.

Table 5: Concentration of identified coumarins in *Peucedanum ostruthium* hairy-root clones (µg·g^{-1} dry weight)

coumarin	L8MS	L8B5	T22HM	T22MS	T22B5	T53B5	T57B5	T55B5
oxypeucedanin					2.8			
imperatorin		3.7	2.3		6.3	2.6	2.1	4.7
isoimperatorin	1.0	8.9			13.0	8.0	7.6	9.2
ostruthin					2.3			

Figure 24 (page 36) shows the chromatograms of hairy-root clone T22 cultured in three different media. Isoimperatorin was found in samples A (medium ½ MS) and C (medium B5). In sample B (medium MS) no coumarins could be identified. Sample C is the only one in which both oxipeucedanin and ostruthin were found.

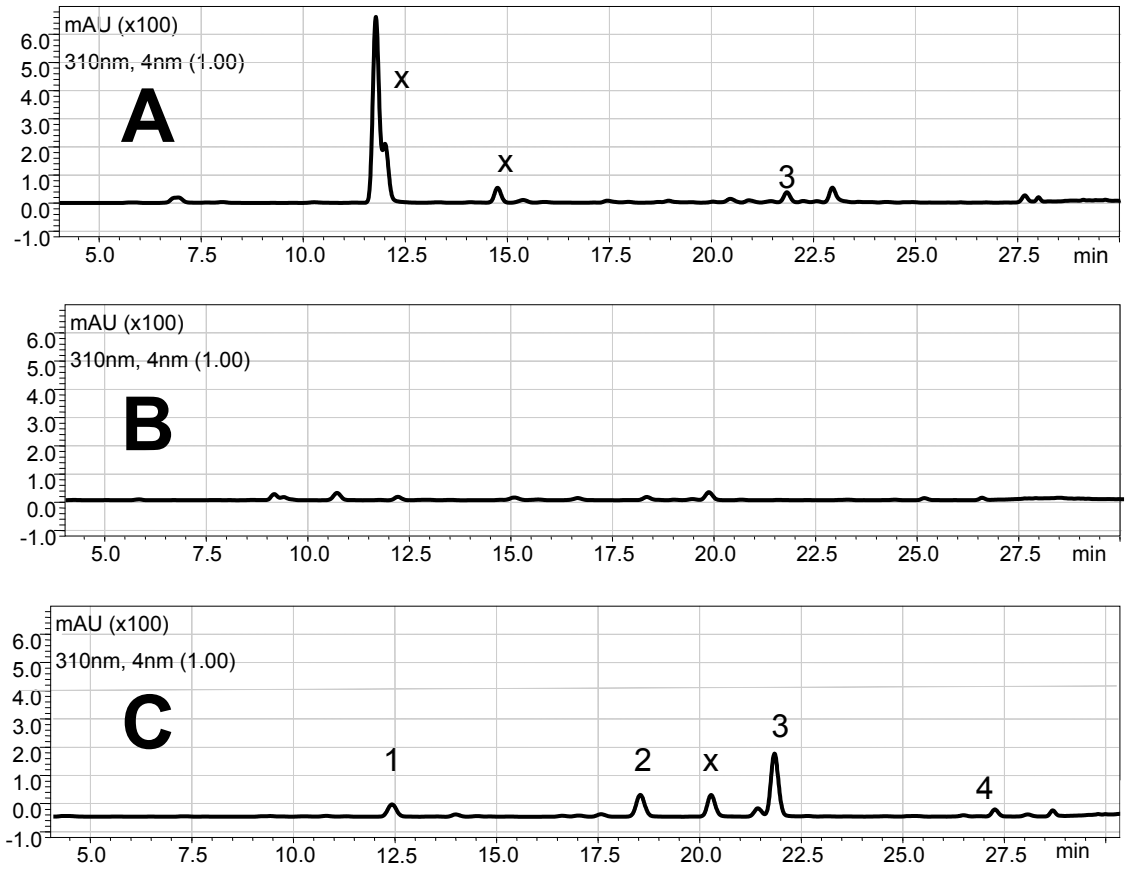

Figure 24: HPLC chromatograms (system see page 18) of *P. ostruthium* hairy-root clone T22 cultivated in medium ½ MS (A), medium MS (B) and medium B5 (C). Peak numbers refer to oxypeucedanin (1), imperatorin (2), isoimperatorin (3), ostruthin (4) and unidentified compound (x)

The nutrient medium definitively had an influence on secondary metabolite formation. Figure 25 on page 37 shows chromatograms of hairy-root clone L8 grown in media MS and B5. More isoimperatorin was found in samples cultured in B5 medium than in those cultivated in MS medium; imperatorin could only be detected in the sample which had been cultivated in medium B5.

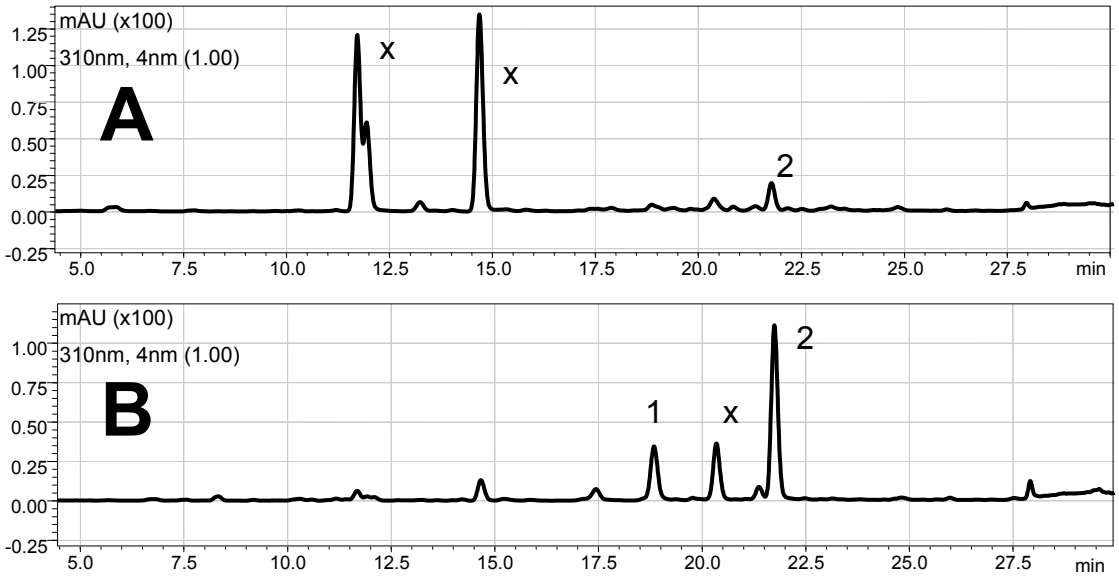

Figure 25: HPLC chromatograms (see page 18) of *P. ostruthium* hairy-root clone L8 cultivated in medium ½ MS (A) and medium MS (B). Peak numbers refer to imperatorin (1), isoimperatorin (2) and unidentified compound (x)

As illustrated in figure 26 (page 38), hairy-root clones cultivated in medium B5 seemed to produce a similar spectrum of secondary metabolites. The peaks with retention times of 18.8 min (imperatorin), 20.3 min (unknown) and 21.7 min (isoimperatorin) were found with a similar concentration in all hairy roots cultured in B5 medium, only in clone T57B5 less imperatorin was detected. The first peak in the chromatogram of clone T22B5 with a retention time of 13.4 min (unknown) was detectable only in this sample. The most prominent peak of the samples cultured in medium B5 was found at the retention time of 21.7 min (isoimperatorin) and was also detected in clones T22HM (cultured in ½ MS medium) and L8MS (cultured in MS medium).

Isoimperatorin was the only detectable compound in the chromatogram of the clone T22HM, cultured in ½ MS medium. Substantial peaks were also found in sample T22HM at retention times of 11.7 min (unknown) and 12.0 min (unknown). These two peaks only occurred in this sample and in clone L8MS (cultured in MS medium), but in a smaller concentration than the clone cultured in ½ MS medium. In addition, for future analyses it is recommended to modify the mobile phase to achieve a better separation of these double peaks.

The two clones cultivated in MS medium revealed a very different spectrum of compounds. The double peak at 11.7 min and 12.0 min (see above) also occurred in sample L8MS, with an additional peak at 14.6 min. Besides, only a small amount of isoimperatorin was detected. In sample T22MS only traces of unknown compounds were found.

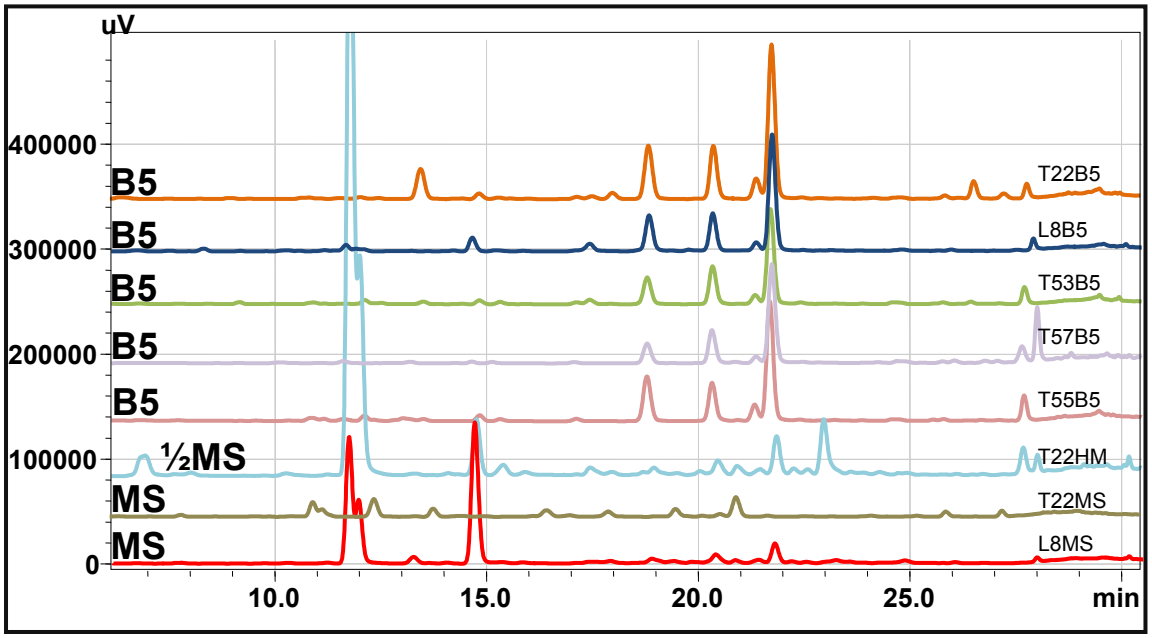

Figure 26: HPLC chromatograms (see page 18) of various hairy-root clones of *Peucedanum ostruthium* cultured in media B5, 1/2 MS and MS

These observations lead to the conclusion that the nutrient medium had a notable influence on the production of secondary metabolites as shown in figure 26. All hairy-root clones cultured in B5 medium showed a very similar chromatogram. Also the hairy-root sample L8B5 established with the *Agrobacterium* strain LBA 9402 showed the same results in cultivation with B5 medium as the hairy-root clone established with TR 105. The clone T22 was cultured in all three media and shows different chromatograms depending on the use of the nutrient medium. This leads to the conclusion that the medium has higher influence on the production of the examined secondary metabolites than the bacterial strain used for the establishment of hairy roots.

4. Discussion

Several studies have revealed that hairy roots tend to grow faster than non-transformed roots, that they are genetically and biochemically stable, and have the ability to synthesize natural compounds as found *in vivo* in normal roots (Christey and Braun 2004; Georgiev et al. 2007; Srivastava and Srivastava 2007). In contrast to numerous studies on Apiaceae plants that have been published so far, the present study is to our knowledge the first to deal with the establishment of hairy roots in *Peucedanum ostruthium.* The aim of this study was to establish an effective transformation system for *Peucedanum ostruthium* to induce hairy-root formation. Furthermore, the effects of the bacterial strains used for transformation, and the effects of the culture medium on the roots' secondary metabolism were examined, the coumarins and furanocoumarins being the center of interest.

These valuable secondary metabolites show antioxidant, antimycobacterial, anticoagulant, antitumoral, antiviral, antifungal and anti-inflammatory activity (Garcia-Argaez et al. 2000; Schinkovitz et al. 2003; Genovese et al. 2009; Riveiro et al. 2010; Thuong et al. 2010).

It is well known that both the starting plant material and *Agrobacterium* strain are critical factors for a successful transformation. Therefore, we firstly used *in vitro*-cultivated plantlets, and leaf explants which were prepared from adult plants growing *in vivo* in the garden of the Department of Pharmacognosy (University of Vienna). Secondly, we performed all infection experiments with three different *A. rhizogenes* wildtype strains. The strains clearly have an influence on the production of secondary metabolites and show different capability of forming hairy roots, as further outlined below.

For the establishment of *in vitro*-cultures of masterwort, seeds were germinated. A quick test, done on wet filter paper in a petri dish, at first indicated a low germination rate. We therefore applied two pretreatments, on the one hand a 24-hour soak on an aqueous solution of gibberellic acid (500 ppm) which is well known to promote seed germination in certain species (Rahnama-Ghahfarokhi and Tavakkol-Afshari 2007). On the other hand, a batch of seeds was stored for 4 weeks at 5°C and then transferred to 25°C.

The cold pretreatment resulted in improved germination and also gibberellic acid had a supportive effect. No further investigations were performed on seed germination as enough seedlings were obtained for the further studies. For the establishment of shoot cultures we used MS medium supplemented with 0.5 µM of the cytokinin benzylaminopurine. This proved to be suitable for satisfactory multiplication, and thus no other growth regulators were tested.

The source, type and age of the explant can influence the transformation effectiveness (Baranski 2008), just as preculture conditions as shown in transformations with the closely related species *Agrobacterium tumefaciens*: The transformation relies on the ability of the bacterium to pass the cuticle, and in prolonged preculture *in vitro* this waxy layer decreases (Tokuji and Fukuda 1999). Our results absolutely confirm these findings: *In vitro* plants or shoots were clearly the appropriate plant material with *Peucedanum ostruthium* and the three *A. rhizogenes* strains that we used.

In our experiments both petioles and leaf veins were chosen as target sites for infection. Studies show that it is much more favorable to use petioles instead of any other plant organs, such as cotyledons, hypocotyls or roots (Pawlicki et al. 1992). The present study for hairy roots in *Peucedanum ostruthium* confirmed the results of Pawlicki (1992), because we obtained the highest hairy-root formation rate by scratching the petioles.

Hairy-root formation could be achieved after transformation of *P. ostruthium* with the *Agrobacterium rhizogenes* wild-type strains TR 105, LBA 9402 and ATCC 15834. However, these strains varied in their effectiveness. Generally, genetic transformation mediated by *Agrobacterium rhizogenes* is affected by several factors like e.g. explant genotype, chemical and physical factors, structure of the explants, and bacterial strain (Tao and Li 2006). Also, the density of the *Agrobacterium* suspension culture is an important factor (Park and Facchini 2000). It is known that different strains of *Agrobacterium rhizogenes* vary in the ability of forming hairy roots (Vanhala et al. 1995; Zehra et al. 1998; Krolicka et al. 2001).

This was confirmed in the present study: Using *Agrobacterium rhizogenes* strains TR 105, LBA 9402 and ATCC 15834, the strain TR 105 was found to be the most virulent one, followed by ATCC 15834. Strain LBA 9402 resulted

in the lowest infection rate. Studies about hairy-root induction in *Linum tauricum ssp. tauricum* with *Agrobacterium* strains TR 105 and ATCC 15834 also showed a higher frequency of hairy-root formation with strain TR 105 (Ionkova and Fuss 2009). In general, signal phenolics like acetosyringone can be used to enhance the hairy-root induction frequency (Gelvin 2000). In future investigations on *P. ostruthium* this might be a promising approach to improve hairy-root induction frequency with less virulent *Agrobacterium rhizogenes* strains like e.g. LBA 9402.

Finally, our results indicate that *P. ostruthium* differs from other species in its specific behavior of forming hairy roots only in complete darkness. For other plants the light conditions are not a critical factor (Hamill and Lidgett 1997). The ½ MS nutrient medium, containing the least amount of nutrients, showed the lowest growth of the hairy-root clones obtained in this study. Hairy roots of *Anethum graveolens,* grown in ½ MS medium and cultured for 30 days, showed a 13-fold biomass increase (Figueiredo et al. 2002). These results clearly exceeded the results gained with the best-growing *Peucedanum* hairy roots.

Studies performed on *Ammi majus* hairy-root cultures established in MS medium demonstrated a 150-fold biomass increase after 30 days of cultivation (Figueiredo et al. 2005). Hairy-root cultures of *Daucus carota* cultured in MS medium showed a 77-fold fresh-weight increase after one month (Pletsch et al. 2002). In our study, a 5-fold fresh-weight augmentation of the fastest-growing hairy-root sample cultured in MS medium for 21 days could be observed. Hairy-root cultures of *Levisticum officinale* showed a 35-fold increase of fresh weight after 30 days grown in B5 medium (Figueiredo et al. 2005) in contrast to the 9-fold biomass increase with clone T51B5 in *Peucedanum ostruthium*.

The nitrogen content of the nutrient medium was evidently essential. With higher nitrogen concentration (½ MS medium → MS medium → B5 medium) hairy-roots growth increased in most hairy-root clones used in this study, independently of the *Agrobacterium rhizogenes* strain used for transformation. The choice of nutrient medium clearly had much more influence on hairy-root growth than the bacterial strain used for

transformation. Nevertheless, different bacterial strains did make some difference in hairy-root growth: Hairy roots established with strain showed the least ATCC 15834, those obtained with strain LBA 9402 a moderate, and TR 105-induced clones the highest increase in growth, when cultivated in medium B5. Hairy roots have varying capabilities of producing growth regulators like auxins and cytokinins by themselves (Guillon et al. 2006). This might be a reason why certain hairy roots obtained through infection with certain *Agrobacterium* strains exhibit better growth than others – both generally, and as observed in our studies.

Hairy-root cultures of several plant species have been studied for the production of phytochemicals like e.g. pharmaceuticals, cosmetics and food additives (Giri and Narasu 2000; Guillon et al. 2006). In the present study, some coumarins and furanocoumarins which are known to occur in the underground organs of *Peucedanum ostruthium* (Vogl et al. 2011) were not detectable in the available hairy-root clones. Only four coumarins out of the seven expected compounds could be clearly identified, but still unknown compounds were detectable. Similarly, in previous studies on the formation of furanocoumarins in hairy roots of *Ammi majus* no furanocoumarins could be detected (Krolicka et al. 2001).

Within further studies the induction of coumarin and furanocoumarin formation by abiotic and biotic elicitors should be examined, because certain elicitors are known to stimulate the production of secondary metabolites in hairy roots of a number of plant species (Guillon et al. 2006). For example, methyl jasmonate can increase the biosynthesis of secondary metabolites without affecting hairy-root growth performance (Pinol et al. 2003; Reddanna et al. 2003; Yaoya et al. 2004). Generally, the production of secondary metabolites may also require interaction between leaves and roots, with e.g. metabolic precursors in roots and bioconversion in leaves (Giri and Narasu 2000).

The amount of coumarins in the hairy-root clones investigated within this study was only approximately 0.001% in comparison to the drug samples of wildly grown *Peucedanum* rhizomes analyzed recently (Vogl et al. 2011). However, some unidentified components showed a typical coumarin-like UV spectrum. These substances seemed to be the main components in the

hairy-root clones. In future studies a HPLC-DAD-MS approach might help to elucidate the secondary metabolism and the unknown compounds in hairy roots of *Peucedanum ostruthium*.

5. Summary

Peucedanum ostruthium (L.) Koch (masterwort) is a widely used plant in traditional Austrian medicine, e.g. in forms of liquor or tea. Coumarins in masterwort rhizomes are of pharmaceutical relevance, as a number of studies have shown antibacterial, antiviral, anti-inflammatory, antifungal, antioxidant and antitumoral activities.

The present thesis deals with the establishment of transformed hairy roots in *Peucedanum ostruthium* under controlled regimes. Multiple shoot cultures were established from aseptically germinated seeds. At this, germination frequency could be improved through a four-week pretreatment of the seeds at 5°C. A second way to increase germination rates is the use of gibberellic acid.

Explants prepared from *in vitro*-cultures as well as from *in vivo*-growing plants were infected *in vitro* with three wildtype strains of *Agrobacterium rhizogenes*, the causative bacterium of the hairy-root disease. Both the type of starting material and the infection technique proved to be significant for a successful transformation. The use of explants from *in vitro*-cultures resulted in good hairy-root formation rates while explants from *in vivo*-growing plants did barely respond to the bacterial infection. Scratching the explants with a scalpel blade dipped in the bacterial suspension proved to be the most suitable technique. Interestingly, no hairy roots were formed under light conditions.

The obtained hairy-root clones differed in their growth rate, depending on the bacterial strain used for infection and the nutrient medium for subsequent cultivation. Clones established with *Agrobacterium* strain TR 105 showed the fastest growth, and for most hairy-root clones B5 medium was best suited in terms of biomass increase.

HPLC-DAD analyses of a number of hairy-root clones revealed that under the given culture conditions definitively less coumarins were formed when compared to non-transformed naturally-grown roots. Also, the coumarin pattern typical for natural roots was not found in any of the eight investigated clones, and the nutrient medium also seemed to influence coumarin

formation. In one sample, none of the seven main coumarins of masterwort could be detected. In the other clones from one to four known compounds (imperatorin, isoimperatorin, ostruthin and oxypeucedanin) were identified.

For future studies it might be considered to investigate the influence of further nutrient media on growth and especially on coumarin formation. In addition, the use of elicitors could possibly also influence coumarin biosynthesis in both quantitative and qualitative ways.

6. Zusammenfassung

Peucedanum ostruthium (L.) Koch (Meisterwurz) findet in Form von alkoholischen Auszügen oder Teezubereitungen verbreitete Anwendung in der österreichischen Volksmedizin. Die Rhizome der Pflanze enthalten pharmazeutisch relevante Cumarine, deren antibakterielle, antivirale, entzündungshemmende, antimykotische, antioxidative und antitumorielle Wirkeigenschaften durch entsprechende Studien belegt sind.

In der vorliegenden Arbeit wurden an *Peucedanum ostruthium* transformierte „hairy roots“ gebildet. Dazu wurden zunächst ausgehend von *in vitro*-gekeimten Samen Sprosskulturen angelegt. Die Samenkeimung konnte durch eine vierwöchige Kältevorbehandlung bei 5°C, und noch mehr durch eine Vorbehandlung mit Gibberellinsäure, gegenüber der Keimung bei Raumtemperatur deutlich verbessert werden.

Es wurden Explantate aus *in vitro*-Kulturen und Freilandpflanzen präpariert und diese dann mit drei Wildstämmen von *Agrobacterium rhizogenes* infiziert. Dieses Bakterium ist Auslöser der „hairy roots“-Krankheit, wobei der Grad der Infektion, gemessen an der Transformationsrate, abhängig vom Explantattyp und von der Infektionstechnik ist: Explantate aus *in vitro*-Kultur reagierten gegenüber jenen aus Freilandpflanzen mit gesteigerter Wurzelbildung, weiters hat sich im Allgemeinen das Anritzen des Explantats mit einer in Bakteriensuspension getauchten Skalpellklinge als bestgeeignete Infektionsmethode erwiesen. Die Kultivierung im Licht brachte bemerkenswerterweise für die Bildung von „hairy roots“ keine Erfolge.

Die erhaltenen „hairy roots“-Klone zeigten in Abhängigkeit von Bakterienstamm und Nährmedium unterschiedliches Wachstum: Klone von Explantaten, welche mit dem *Agrobacterium*-Stamm TR 105 infiziert worden waren, wiesen das beste Wachstum auf, außerdem führte der Einsatz des Nährmediums B5 zum höchsten Zuwachs an Biomasse.

Erste HPLC-Analysen einiger „hairy roots“-Klone zeigten, dass unter den gegebenen Kulturbedingungen deutlich weniger Cumarine gebildet wurden als in natürlich gewachsenen, nichttransformierten Wurzeln. Keiner der acht untersuchten Klone wies das für die Meisterwurz typische Cumarinmuster

auf, und auch das Nährmedium schien Einfluss auf die Cumarinbildung zu haben. In einem Klon konnte keines der sieben Hauptcumarine der Meisterwurz detektiert werden, in den restlichen Klonen waren ein bis vier Cumarine (Imperatorin, Isoimperatorin, Ostruthin und Oxypeucedanin) nachweisbar.

In weiterführenden Studien sollte der Einfluss anderer Nährmedien auf das Wachstum und vor allem auf die Cumarinbildung untersucht werden. Außerdem könnte auch der Einsatz von Elizitoren quantitative und qualitative Auswirkungen auf die Cumarinbiosynthese haben.

7. References

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