Division of Pharmaceutical Biology
Faculty of Pharmacy
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Plant secondary metabolites in *Peucedanum palustre* and *Angelica archangelica* and their plant cell cultures

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ACADEMIC DISSERTATION

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Конéцъ , и бгу̀ слáва

2 ABSTRACT

A reversed-phase, high-performance liquid chromatographic method (RP-HPLC) with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) detection was developed utilising Turbo Method Development® and DryLab® programmes for the separation and identification of coumarins in *Peucedanum palustre* L. (Moench) and *Angelica archangelica* (L.) var. *archangelica*. Fifteen coumarins were identified both in *P. palustre* and in *A. archangelica*. This is the first report on the xanthotoxin, isopimpinellin, pimpinellin, and coumarin composition of the umbels of *P. palustre.*

The coumarin composition of Finnish *P. palustre* populations was analyzed and verified chromatographically. The main coumarin in roots was oxypeucedanin, and in aerial parts peulustrin/isopeulustrin. The highly varying total coumarin concentration was the highest in umbels and the lowest in stems. Leaves and roots contained comparable amounts of coumarins. The total coumarin concentration decreased towards the north. As regards the aerial parts, the coumarin content of the umbels and leaves resembled each other the most. The effective temperature sum clearly correlated with the coumarin concentrations of the aerial parts, but not with the roots of the plant. The study did not support the existence of chemotypes in Finnish *P. palustre* populations.

A spontaneously embryogenic cell line of *A. archangelica* was established from seedlings *via* callus formation. The highest coumarin production was achieved after three weeks of cultivation in the medium containing 3.0% sucrose. Cryopreservation was found to be a suitable method for storing the cell line. Plantlets propagated in an air-sparged bioreactor were transferable directly to soil. The coumarin composition and levels in the regenerated plants were comparable to those in intact plants.

A mathematical computer-aided model CELLOP was constructed in which the desirability functions in a three-dimensional experimental design are used for optimising the growing conditions for plant cultures. The calcium, inorganic nitrogen, and sucrose concentrations in the medium were optimised for coumarin-producing, spontaneously embryogenic cell lines of *A. archangelica* and *P. palustre*. In comparison to the reference, the dry mass for *A. archangelica* was 24.7% and the coumarin concentration 40.5% higher in the optimised conditions, and the dry mass for *P. palustre* 61.8% and the coumarin concentration 58.1% higher. For *A. archangelica* the highest embryogenic activity occurred in the medium containing 1.25 mM calcium and for *P. palustre* in the medium containing 50.0 mM NO_3^- and 4.01 mM NH_4^+.

3 LIST OF ORIGINAL PUBLICATIONS

I Eeva, M., Rauha, J.-P., Vuorela, P., Vuorela, H.: Computer-assisted, High-performance liquid chromatography with mass spectrometric detection for the analysis of coumarins in *Peucedanum palustre* and *Angelica archangelica*. - Phytochem. Anal. **15**: 167-174, 2004.

II Eeva, M., Yrjönen, T., Summanen, J., Vuorela, P., Vuorela H.: Variability in coumarin composition of *Peucedanum palustre* in Finland. - submitted to J. Chem. Ecol.

III Eeva, M., Ojala, T., Tammela, P., Galambosi, B., Vuorela, H., Hiltunen, R., Fagerstedt, K., Vuorela, P.: Propagation of *Angelica archangelica* plants in an air-sparged bioreactor from a novel embryogenic cell line, and their production of coumarins. - Biol. Plant. **46**: 343-347, 2003.

IV Eeva, M., Vuorela, P., Tammela, P., Nyman, M., Ojala, S., Fagerstedt, K., Haario, H., Vuorela H.: Development of the CELLOP optimisation model for plant cell cultivation. - Biol. Plant. **51**: 27-33, 2007.

These publications will be referred to in the text by their Roman numerals

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4 ABBREVIATIONS

ABA	abscisic acid
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
DAD	diode array detector
DM	dry mass
FM	fresh mass
GA	gibberellic acid
HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole butyric acid
JA	jasmonic acid
KIN	kinetin
LC/MS	liquid chromatography/mass spectrometry
MeJa	methyl jasmonate
MeOH	methanol
MS	mass spectrometry
MW	molecular weight
Ph.	Pharmacopoea
RP-HPLC	reversed phase high performance liquid chromatography
TLC	thin layer chromatography
UV-A	ultraviolet A light, long wave
XIC	extracted ion chromatogram/current

5 INTRODUCTION

Angelica (*Angelica archangelica* L. subsp. *Archangelica*) is naturally distributed throughout Northern Europe and Eastern Siberia, and is cultivated in Hungary, France, the Netherlands, Belgium and Finland for food and liqueur production. The umbelliferous plant *Peucedanum palustre* (L.) Moench is widespread in Europe (Meredith and Grubb 1993). It belongs to the endemic flora of Finland and is relatively common in southern Finland and frequently found along the south coast of Finland. It is less common in northern Finland. It grows in moist inland environments, on riverbanks or in moist forests (Hämet-Ahti *et al.* 1998). Both plants contain essential oils (Forsen 1979, Nykänen *et al.* 1991, Schmaus *et al.* 1989) and coumarins (Murray *et al.* 1982*,* Vuorela *et al.* 1988, Vuorela *et al.* 1989*,* Hadaček 1989, Härmälä 1991). The coumarins are benzo-*α*-pyrones, lactones of 2-hydroxy-*Z*-cinnamic acids (Bruneton 1999). The coumarins in *P. palustre* and *A. archangelica* are either simple coumarins, hydroxylated, alkoxylated, and alkylated derivatives of the parent compound, coumarin, together with their glycosides (Murray *et al.* 1982) or furanocoumarins with a five-membered furan-ring attached to the coumarin nucleus. Furanocoumarins are divided into linear and angular furanocoumarins with substituents at one or both of the remaining benzenoid positions. Coumarins possess several biological activities, but they also play an important role in defence mechanisms against insects and plant pathogens (*e.g.* Hadaček *et al.* 1994*,* Ojala *et al.* 1999*,* Siskos *et al.* 2008). They also act as attractants to certain insect species (Meredith and Grubb 1993, Stanjek *at al.* 1997).

The basic methods used for plant coumarin analysis are thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Vuorela 1988*,* Hadaček 1989*,* Härmälä 1991*,* Hadaček *et al.* 1994*,* Roos *et al.* 1997*,* Zschocke *et al.* 1998*,* Hawryl *et al.* 2000). The traditional analytical methods require large amounts of sample material and laborious sample preparation, and are thus suitable for only a limited number of samples. Due to the complex coumarin composition of umbelliferous plants, the chromatographic separation of closely related compounds is also often unsatisfactory. Coupling HPLC with mass spectrometry (MS) enables the differentiation of unresolved peaks, and is therefore well suited for the analysis of samples containing closely related compounds. Some studies involving application of the LC/MS technique to the coumarin analysis of plants of the *Angelica* family have been published. Zschocke *et al.* (1998) investigated the coumarin composition of *Angelica sinensis* (Oliv.) Diels root, and Roos *et al.* (1997) coumarins from *Angelica archangelica* L.. Computer-based optimisation methods, such as Turbo Method

Development® and DryLab®, facilitate plant sample analysis by markedly reducing the number of initial runs needed. However, this approach has rarely been used for the development of a LC/MS method suitable for the rapid analysis of a large number of samples.

Several research groups have studied the coumarin composition of *P. palustre*, but very little quantitative data are available. Only one report involving a more comprehensive analysis of the quantitative coumarin composition of *P. palustre* roots, stems, leaves, and fruits has been published (Hadaček, 1989). The coumarin composition of root samples of a number of *Peucedanum* species was analyzed in this study, thereby enabling a more accurate systematic classification of the genus. In addition, comparative HPLC analyses of four *Peucedanum* species, including also *P. palustre*, have been carried out to investigate the quantitative differences in coumarins in different plant organs within the same plant species. A total of seven coumarins were detected, the linear furanocoumarins oxypeucedanin, ostruthol, and oxypeucedanin hydrate, and the angular dihydrofuranocoumarin columbianadin, being the major coumarins in the Austrian *P. palustre*. The results also revealed differences in the coumarin composition of the individual plant organs.

Somatic embryogenesis makes it possible to produce plant material to be used not only in developmental studies but also in plant propagation. Somatic embryogenesis was reported for the first time in *Oenanthe aquatica* L. (Waris 1957) and in *Daucus carota* L. (Reinert 1958, Steward *et al.* 1958). Since then, somatic embryogenesis has been reported in at least 30 Apiaceae species. As the germination ability of *A. archangelica* seeds is very low (Ojala 1985), alternative propagation methods are needed. The maintenance of cell cultures is often impractical and may lead to loss of the desired characteristics, *e.g.* ability for embryogenesis. Thus a preservation method, such as cryopreservation, offers the possibility for long-term storage of a plant cell line (Cho *et al.* 2000).

Secondary metabolite production may be increased by means of genetic engineering, but growth medium optimisation is still needed in the development of a feasible bioprocess (Verpoorte *et al.* 2002). Traditionally the effect of a single explanatory variable on the whole system has been determined by omitting the interactions of the other nutrients. Thus optimisation methods must be developed that enable fast, reliable approximation of the interactions between several variables in a multivariable system. Minor modification to a standard media, such as Murashige and Skoog (1962) or Gamborg B5 (Gamborg *et al.* 1968), is a common approach when establishing a new cell culture. A more recent approach is to optimise the growing medium of the *in vitro* culture by analysing the mineral proportions of the intact plant material (Gonçalves *et al.* 2005). A more comprehensive approach is needed

in order to understand the complexity of the requirements of a novel culture, as well as to reduce the number of experiments and the time required. Systematic optimisation is frequently used for microbial cultures, and is also applied to plant cell cultures. A widely used design has been the central composite design (Eilers *et al.* 1988, Tuominen *et al.* 1989, Nuutila *et al.* 1991, Toivonen *et al.* 1991, Chattopadhyay *et al.* 2003) developed by Cochran and Cox (1957), which employs regression analysis. Response surface methodology is applied to map the expected performance of a range of response variables in a multi-variable system. In these studies, interactions between variables markedly affected the system. Methods designed for slowly growing cultures based on several initial experimental starting points have also been published (Tammisola *et al.* 1993), enabling a more simultaneous approach and making it easier to avoid time-dependent variables such as seasonal or even genetic changes. "PRISMA", a prismatic solvent mixture design model for analytical purposes developed by Nyiredy *et al.* (1991), combined with desirability functions by Deming (1991), have been successfully used in HPLC optimisation (Outinen *et al.* 1998). These enable combination of a medium mixture design based on "PRISMA" and statistical evaluation and modelling of the response variables by means of desirability functions for predicting the behaviour of the response variables, *i.e.* the desired features of a novel plant cell culture.

6 REVIEW OF THE LITERATURE

6.1 Botany and distribution of *A. archangelica* and *P. palustre*

***A. archangelica* L.** Apiaceae (Umblliferae) is a robust monocarpic perennial growing 1.5 - 2 m tall. The plant is characterized by a spindle-shaped, thick root with many long, descending rootlets. The basal leaves are large (up to 80 cm) and petiolate, with petiole sheaths conspicuously inflated and blades triangular in outline, bi- to tripinnate. The leaflets are large and wide with finely serrated edges. The upper alternate leaves have a strongly inflated petiole, and laminae are small or absent. Bracts are absent and bracteoles are mainly linear. The stalk is erect, striate, hollow, and branchy, and has distinctive reddish to violet colour. The primary umbel has 1-3 early withering sheaths, the secondary umbels are spherical, and the sheaths are linear and declined. Fruits are dorsally flat, elliptic to oblong with winged lateral ridges. The apex leaflet is usually unlobed with a petiole concave in cross-section. Robust petioles are cylindrical in cross-section, leaflets unevenly serrated, and

lobed. The leaf apex is 3-lobed, the corolla is white to greenish, and the lateral ribs of the fruits are thick.

The species is usually divided into two subspecies: *A. archangelica* L. subsp. *litoralis* (Fr.) Thell. and *A. archangelica* L. subsp. *archangelica*. The pedicles of subsp. *litoralis* (Fr.) Thell. are glabrous and the sheaths of the secondary umbels are approximately one half the length of the pedicle. The fruits are 5-6 x 3.5-4.5 mm of size, ovate and have a pungent odour. The dorsal ribs are low and blunt. The species grows on open, rocky seashores of northern Europe (Hämet-Ahti *et al.* 1998). Pedicles of the subsp. *archangelica* are hairy to sub-glabrous and the secondary umbel sheaths are of the same length as the pedicle. The fruits are 6-8 x 4-5 mm long, oblong, with a pleasant odour, and the dorsal ridges are prominent and acute. It grows on creek and river banks in moist inland environments or in moist forests, especially in the fell region in northern Finland. The subsp. *archangelica* is divided into three varieties: var. *archangelica*, var. *decurrens* (Ledeb.) Weinert, and var. *sativa* (Mill.) Rikli (Weinert 1973). The populations of subsp. *archangelica* in Finland and Scandinavia are treated as var. *archangelica*. Both the odour and taste of the fruits are pleasantly aromatic. The plant blooms from June to August, depending on the location (Hämet-Ahti *et al.* 1998).

In Finland, *A. archangelica* has scattered occurrences in Inari and Enontekiö in Lapland, and further south it mainly grows along the River Ounasjoki in the area of the Kemijoki river system. A disjunctive occurrence has also been found at Somerniemi in South Finland. It has also been cultivated in South Finland at Puumala and Turku. *Angelica* is also cultivated in Europe, especially in Germany, Belgium, and France (Hoppe 1975, Ojala 1984, Hämet-Ahti *et al.* 1998). The growing area of *Angelica archangelica* (L.) *subsp. archangelica* extends from Finland and Scandinavia to East Siberia, the southern limit of distribution running from central Germany to Altai and Lake Baikal. There is a large discrete distribution area in the Himalayas, and the plant also occurs in Greenland, Iceland, and the Faeroe Islands (Weinert 1973).

***Peucedanum palustre* (L.) Moench** Apiaceae (Umblliferae) is in English called hog's fennel, marsh parsley, or milk parsley due to the white, sticky fluid exuding from the broken tissues of young plants. The herb is almost sub-glabrous, and in Finland it grows 50-100 cm tall. It is usually described as annual or biennial, but also sometimes as perennial. The taproot is simple, spindle-shaped and up to 20 cm long with slender laterals up to 10 cm long. The stems are sulcate, and hollow. The basal leaves are 2 to 4-pinnate, triangular in outline, and the lobes are 5-10 mm long, ovate in outline, and pinnately lobed. The ultimate lobes are

linear or oblong, entire or 2 to 3-fid, with an obtuse cartilaginous apex and black tips. The plants have 1-8 lateral flowering branches with 20-40 rays which are puberulent on the inner side. The petioles, 2-3 mm wide, are canaliculated and expanded at the base. The bracts and bracteoles are very unequal, lanceolate, sometimes 2 to 3-fid, and deflexed. The flowers are 2-3 mm in diameter with ovate, obtuse sepals. The petals are white and papillose-puberulent above. The elliptical fruit is 4-5 mm long, the wing is 0.5 - 0.75 mm wide, the dorsal ridges are wide and prominent, and the commissural resin canal is concealed by the pericarp (Hämet-Ahti *et al.* 1998, Meredith and Grubb 1993).

P. palustre grows in wet places such as swampy meadows and marshy soils in fens, fen scrub, fen woodland, at the waterfront of ponds, in wet spruce swamps, and ditches, but also in cliff cracks and swampy low-lying cliff formations in the outer archipelago, the plant being common throughout the whole of Finland apart from northern Lapland (Hämet-Ahti *et al.* 1998). In Europe the species ranges from Britain and France to the Urals and Altai in central Asia, and from Finland and Scandinavia to Italy, the Balkans, and Bulgaria. It is absent from the Iberian Peninsula, Albania, Greece, and Turkey. It extends very widely through both the deciduous forest zone and the boreal zone. In western parts of Germany the species is very widespread in the northern third and in the valleys of the southern third, but generally very local in the central third of the country (Meredith and Grubb 1993, Hämet-Ahti *et al.* 1998).

6.2 Ethnobotany of *A. archangelica* and *P. palustre*

Angelica herb, seeds, and roots have been used for centuries in folk medicine and especially as a food ingredient and a vitamin source by the Same, the inhabitants of the Faeroe Islands, and other Nordic peoples (Bergius 1782). In Viking times angelica root was a major export commodity from Scandinavia, especially from Norway to the continent. The Same used angelica against coughs and the leaves topically to relieve oedemas. The stems collected prior to flowering, candied with sugar, were also used as a form of candy. It was primarily used for medicinal purposes, being supposed to cure almost every disease including the plague (Jóhansen 1994).

The printed history of angelica begins in the 17th century with the pharmacopoeia of Stockholm (*Ph. Holmiensis* 1686). Angelica root and seeds are mentioned several times in various compositions and it was one of the main ingredients of theriac, a cure against innumerable ailments. The pharmacopoeic history of angelica in Finland ends with the first edition of *Ph. Fennica* from the year 1819. Still in 1829, angelica was mentioned in 41

European pharmacopoeias (*Ph. Universalis* 1829). Bergius (1782) recommended angelica for its poison-resisting properties, for being beneficial to the stomach, for causing sweating, and for relieving flatulence. Björnlund (1797) mentioned it, in addition as a remedy for swelling or inflammation, also for *"asthma pituitosum, colica frigida, obstructio mensium"*. Later sources mention angelica as a remedy for gastric disorders (Hoppe 1975), lack of appetite, insomnia, rheumatism, and for cough (Blaschek *et al.* 1998). Nowadays it is used as a flavouring agent in medicines and liqueur production.

The ethnopharmacologial history of *Peucedanum palustre* L. (Moench) is somewhat more obscure because *P. palustre* was seldom used in folk medicine. According to Schmaus *et al.* (1989), it was used against pertussis and spasms and, According to Hoppe (1975), it was used against cough, cramps, epilepsy, and gastrointestinal disorders. Other *Peucedanum* species have also been used against rheumatism (Hiermann and Schantl 1998) or gout (Hsiao *et al.* 1998). The sources from the 19th century use several synonyms of the drug and most probably several closely related species of the Apiaceae family have been used for the same purposes. In *Ph. Universalis* (1829) the names *Peucedanum officinale* L. and *Foeniculum porcinum* are mentioned, and in Geiger and Mohr (1845) the drugs *radix Olsnitii* or *Thysselini, radix Apii silvestris, radix Mei palustris, radix Selini palustris L.,* and *Thysellini palustris Hoffm.*. Kubetczka *et al.* (1989) quoted Chamberlain's Flora of Turkey, stating that "it seems unlikely that *Peucedanum* represents a natural assemblage of species; it is more probably a collection of taxa brought together by exclusion from other genera". This gives a good insight into the difficulties in distinguishing between the different species of Apiaceae, which closely resemble each other.

According to *Ph. Universalis* (1829) and Geiger and Mohr (1845), the drug was used for swelling or inflammation, as a diuretic, for stimulating blood flow in the pelvic area and uterus, and against poisoning. The drug was an appreciated remedy against epilepsy. Bergius (1782) recommends it against hysteria, diuretic or for stimulation of the blood flow in the pelvic area and uterus. Some of the old names, *i.e.* Finnish, Swedish, or Russian ginger, indicate that it has been used as spice due to its spicy, but somewhat mordant taste. The Sami have chewed the roots as a substitute for tobacco (Palmstruch 1809).

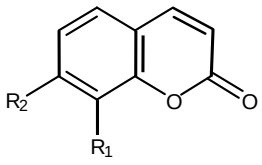
6.3 Phytochemistry and analytics of coumarins

The Tupi Indian word, "coumarou", for coumarin-containing *Dipteryx odorata* (Aublet) Willd. Fabaceae has given the name to the whole group of substances (Bruneton

1999). Coumarins are benzo-*α*-pyrones, lactones of 2-hydroxy-*Z*-cinnamic acids (Bruneton 1999, Matern *et al.* 1999, Borges *et al.* 2005). Coumarin-structured compounds are widely distributed in the plant kingdom, but also present in fungi and bacteria (Matern *et al.* 1999). Already in 1982 Murray listed approximately 1250 monomeric coumarin derivatives isolated from plant sources. Within higher plants, simple coumarins occur in about 70 plant families. Since then, hundreds of new coumarins have been identified (Murray 1989, Murray 1991, Murray 1995, Murray 1997, Borges *et al.* 2005). Coumarins are found free or as heterosides in many dicotyledonous families, such as Apiaceae, Asteraceae, Fabiaceae, Moraceae, Rosaceae, Rubiaceae, Rutaceae, and Solanaceae (Matern *et al.* 1999). Coumarins with an additional ring system, furano- or pyranocoumarins, only occur in 15 plant families, the majority of them being Apiaceae and Rutaceae.

The coumarins present in *P. palustre* or *A. archangelica* can be divided into simple coumarins, (**Figure 1**) hydroxylated, alkoxylated, and alkylated derivatives of the parent compound, coumarin, together with their glycosides (Murray *et al.* 1982). Furanocoumarins have a five-membered furan ring attached to the coumarin nucleus. They are divided into linear (**Figure 2**) and angular coumarins (**Figure 3**), with substituents at one or both of the remaining benzenoid positions. According to Wagner *et al.* (1984), angelica root contains 0.001 - 0.008% coumarins. Gawron and Głowniak (1987) reported coumarin concentrations in angelica fruits of up to 3.5%. A total of 32 coumarins have been reported to be present in *A. archangelica* (**Table 1**) and 20 in *P. palustre* (**Table 2**).

Simple coumarins	**R1**	**R2**
Umbelliferone	H	-OH
Osthenol	$-CH_2-CH=C(CH_3)_2$	-OH
Osthol	$-CH_2-CH=C(CH_3)_2$	$-OCH_3$
Umbelliprenin	H	D



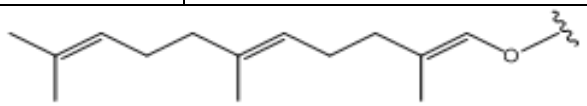


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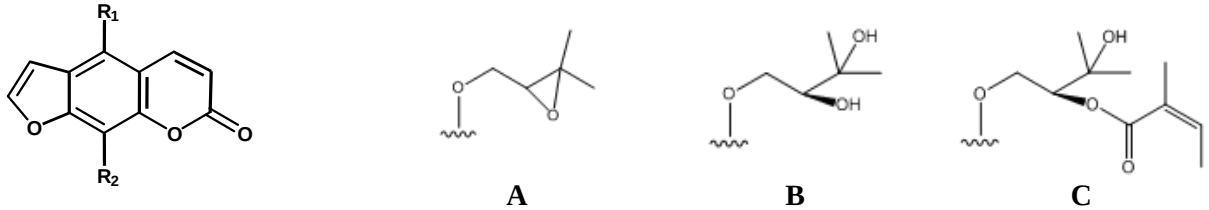
Figure 1. Simple coumarins found in *A. archanigelica* and *P. palustre* in this study.

Böcker and Hahn (1911) isolated a *γ*-lactone from the volatile oil of angelica by steam distillation, which Späth and Pesta (1934) later showed to be osthol that is also found in *Peucedanum ostruthium* L.. Baerheim Svendsen (1954) reported archangelicin, osthenol, umbelliferone, angelicin, archangin, and osthol to be present in *A. archangelica* L. subsp. *norvegica* (Rubr.) Nordh utilising methods such as isolation, adsorption chromatography,

chemical analysis, and paper chromatography. Fisher and Baerheim Svendsen (1976) also found oroselone and apterin in angelica. Späth and Pesta (1939) and Cisowski *et al.* (1987) studied the coumarins in the fruits, roots, and stems of *A. archangelica* L. and *A. litoralis* Fries. They reported that the roots and fruits of both plants contain 13 coumarins, and the stems eight free coumarins.

Angelica root oil has been analysed in detail (Taskinen and Nykänen 1975, Nykänen *et al.* 1991). Forsen (1979) studied the components of the essential root oil during the growing period with a strain from northernmost Finland and *A. archangelica* var. *sativa* of German origin cultivated under the same conditions. The *A. archangelica* var. *archangelica* provided better flavouring properties than *A. archangelica* var. *sativa*, possibly due to differences in the monoterpene composition of the strains. Ojala *et al.* (1986) studied the essential root oil of angelica in 15 populations. One marked feature was an increase in the concentration of 3-carene from the south towards the north.

Linear furanocoumarins	**R1**	**R2**
Psoralen	H	H
Xanthotoxol	H	-OH
Xanthotoxin	H	$-OCH_3$
Bergapten	$-OCH_3$	H
Isopimpinellin	$-OCH_3$	$-OCH_3$
Imperatorin	H	$-O-CH_2-CH=C(CH_3)_2$
Isoimperatorin	$-O-CH_2-CH=C(CH_3)_2$	H
Oxypeucedanin	A	H
Phellopterin	$-OCH_3$	$-O-CH_2-CH=C(CH_3)_2$
Oxypeucedaninhydrate	B	H
Ostruthol	C	H
Isobyakangelicin angelate	C	$-OCH_3$



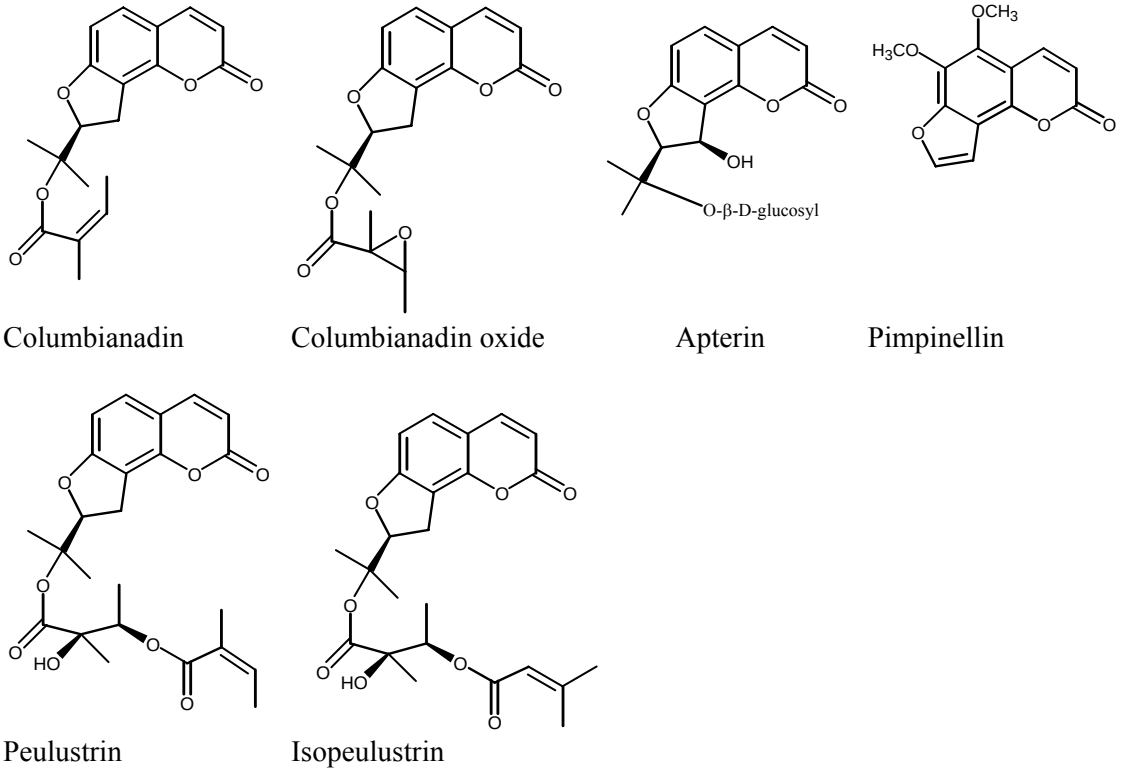
A **B** **C**

Figure 2. Linear coumarins found in *A. archanigelica* and *P. palustre* in this study.

The coumarins reported in *P. palustre* are listed in **Table 2**. The roots have been shown to contain columbianadin, isoimperatorin, ostruthol, (+)-oxypeucedanin, (+)-oxypeucedanin hydrate (Eichstedt Nielsen and Lemmich 1964), peulustrin (Eichstedt Nielsen and Lemmich 1965A), and isobyakangelin angelate (Vuorela *et al.* 1988). The fruits, according to Eichstedt

Nielsen and Lemmich (1965B), contain columbianadin, columbianadinoxide, isoimperatorin, iso-oxypeucedanin hydrate, isopeulustrin, (+)-oxypeucedanin, and umbelliprenin. Bieganowska and Głowniak (1988) reported that the extracts of fruits, herb and roots contained a similar range of coumarins, the main components being columbianadin, imperatorin, isoimperatorin, oxypeucedanin, and oxypeucedanin hydrate. Hadaček (1989) reported that oxypeucedanin hydrate, oxypeucedanin, isobyakangelicin angelate, ostruthol, peulustrin, columbianadin, and isoimperatorin occurred in the fruit, subterraneous parts, leaves, and stems.

The volatile oils of the fruit and stalk of *P. palustre* consists almost entirely of monoterpenes hydrocarbons, and the essential leaf oil of monoterpene hydrocarbons (47%), sesquiterpene hydrocarbons (38%), aliphatic alcohols, and esters (7%), as well as traces of oxygenated sesquiterpenoids. The root oil mainly consists of mono- and sesquiterpenoids (Schmaus *et al.* 1989).



Columbianadin | Columbianadin oxide | Apterin | Pimpinellin

Peulustrin | Isopeulustrin

Figure 3. Structure of the angular coumarins found in *A. archanigelica* and *P. palustre* in this study.

Table 1. Coumarins reported in *Angelica archangelica* L. (F = fruit, L = leaf, R = root)

Compound	**Plant organ and reference**
Angelicin	(F) Corcilius 1956 (L) Steck and Bailey 1969
2'-Angeloyl-3'-isovaleryl vaginate	(R) Härmälä 1991
Apterin	(R) Fischer and Baerheim Svendsen 1976
Archange1icin	(R) Nogushi and Kawanami 1940
Archangelin	(R) Chatterjee and Sen Gupta 1964
Archangin	(R) Baerheim Svendsen 1954
Bergapten	(R) Späth and Vierhapper 1939, Härmälä 1991, **(I)** (L) Steck and Bailey 1969, **(I)** (F) Corcilius 1956
Byakangelicin angelate	(R) Härmälä 1991
Byakangelicin-2'-O-isovalerate	(R) Sun and Jakupovic 1986
Heraclenol-2'-O-isovalerate	(R) Sun and Jakupovic 1986
Heraclenol-2'-O-seneciote	(R) Sun and Jakupovic 1986
8-Hydroxybergapten	(F) Patra *et al.* 1976
Imperatorin	(F) Späth and Vierhapper 1937 (L) Steck and Bailey 1969 (R) Corcilius 1956, Härmälä 1991, **(I)**
Isobergapten	Chatterjee & Dutta 1968
Isoimperatorin	(F) Chatterjee *et al.* 1967 (L) Chatterjee *et al.* 1967 (R) Chatterjee *et al.* 1967, Härmälä 1991, **(I)**
Isopimpinellin	(F) Patra *et al.* 1976 (L) Steck and Bailey 1969, **(I)** (R) Härmälä 1991, **(I)**
5-Methoxy- heraclenol isovalerate	(R) Sun and Jakupovic 1986
8-[2-(3-Methyl- butyroxy)-3-hydroxy-3-methylbutoxy] - psoralen	(R) Härmälä 1991
Oroselone	(F) Baerheim Svendsen 1954 (R) Baerheim Svendsen 1954
Osthenol	(F) Corcilius 1956 (R) Böcker and Hahn 1911, **(I)**
Osthol	(F) Böcker and Hahn 1911, Corcilius 1956 (R) Härmälä 1991, **(I)**
Ostruthol	(F) Chatterjee *et al.*1967 (R) Chatterjee and Dutta 1968, Härmälä 1991, **(I)**
(+)-Oxypeucedanin	(F) Chatterjee *et al.* 1967 (L) Steck and Bailey 1969, I (R) Chatterjee and Dutta 1968, Härmälä 1991, **(I)**
(+)-Oxypeucedanin hydrate	(F) Chatterjee *et al.* 1967 (R) **(I)**
Oxypeucedanin methanolate	(R) Vishwapaul and Weyerstahl 1987
Phellopterin	(F) Beyrich 1965 (R) Härmälä 1991, **(I)**
Pimpinellin	(F) Cisowski *et al.* 1987
Psoralen	(R) Härmälä 1991, **(I)**
Umbelliferone	(F) Sommer 1859 (R) Sommer 1859, **(I)**
Umbelliprenin	(F) Späth and Vierhapper 1938 (R) Corcilius 1956
Xanthotoxin	(F) Späth and Vierhapper 1938 (L) **(I)** (R) Corcilius 1956, Härmälä 1991, **(I)**
Xanthotoxol	(F) Späth and Vierhapper 1937 (R) Corcilius 1956, **(I)**

Table 2. Coumarins reported in *Peucedanum palustre* (L.) Moench. (F = fruit, L = leaf, S = stem, R = root, U = umbel)

Compound	**Plant organ and reference**
Aesculetin	(L) Meredith and Grupp 1993
Apterin	(U) **(I)**
Bergapten	(R) Leskova and Ananichev 1969, Vuorela 1988
Columbianadin	(F) Eichstedt Nielsen and Lemmich 1965B, Hadaček 1989 (R) Eichstedt Nielsen and Lemmich 1964, Vuorela 1988, **(I)** (U) **(I)**
Columbianadin oxide	(F) Eichstedt Nielsen and Lemmich 1965B (R) **(I)** (U) **(I)**
Imperatorin	(F) Leskova and Ananichev 1969 (R) Leskova and Ananichev 1969, Vuorela 1988, **(I)** (U) **(I)**
Isobyakangelicin angelate	(F) Hadaček 1989 (L) Hadaček 1989 (S) Hadaček 1989 (R) Vuorela 1988, Hadaček 1989, **(I)** (U) **(I)**
Isoimperatorin	(F) Eichstedt Nielsen and Lemmich 1965B (R) Eichstedt Nielsen and Lemmich 1964, Vuorela 1988, Hadaček 1989, **(I)** (U) **(I)**
Isooxypeucedanin	(F) Eichstedt Nielsen and Lemmich 1965B
Isopeulustrin	(R) **(I)** (U) **(I)**
Isopimpinellin	(R) **(I)** (U) **(I)**
Ostruthol	(F) Hadaček 1989 (L) Hadaček 1989 (R) Eichstedt Nielsen and Lemmich 1964, Vuorela 1988, Hadaček 1989, **(I)** (S) Hadaček 1989 (U) **(I)**
(+)-Oxypeucedanin	(F) Eichstedt Nielsen and Lemmich 1965B, Hadaček 1989 (L) Hadaček 1989 (R) Eichstedt Nielsen and Lemmich 1964, Vuorela 1988, Hadaček 1989, **(I)** (U) **(I)**
(+)-Oxypeucedanin hydrate	(R) Eichstedt Nielsen and Lemmich 1964, Vuorela 1988, Hadaček 1989, **(I)** (U) **(I)**
Peucedanin	(F) Leskova and Ananichev 1969 (R) Leskova and Ananichev 1969
Peulustrin	(F) Eichstedt Nielsen and Lemmich 1965B, Hadaček 1989 (L) Hadaček 1989 (R) Eichstedt Nielsen and Lemmich 1965A, Hadaček 1989, **(I)** (S) Hadaček 1989 (U) **(I)**
Pimpinellin	(R) I
Umbelliferone	(L) Meredith and Grupp 1993
Umbelliprenin	(F) Eichstedt Nielsen and Lemmich 1965B (R) **(I)** (U) **(I)**
Xanthotoxin	(R) **unpublished data**

Several planar, column, and gas-liquid chromatographic, countercurrent partition and gel filtration methods have been used in the isolation and analysis of coumarins (Murray *et al.* 1982, Erdelmeier 1983). The retention behaviour of coumarins in normal-phase high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) has been studied by Głowniak and Bieganowska (1985) and Bieganowska and Głowniak (1988). Some studies involving the application of HPLC-MS to the analysis of coumarins in plants of the *Angelica* family have also been published. Coupling HPLC with MS rather than with traditional detectors in order to enable differentiation of unresolved peaks is advantageous in the analysis of samples containing closely related compounds. Zschocke *et al.* (1998) investigated the coumarin composition of the roots of *Angelica sinensis*, and Roos *et al.* (1997) studied the coumarins from *A. archangelica*. L. (*Angelica officinalis* Hoff.). In these studies 11 to 16 coumarins were separated and identified.

6.4 Pharmacology of coumarins

In North America cattle fed with hay containing sweet clover (*Melilotus* sp) stored in unfavourable conditions were dying from a hemorrhagic disorder (Schofield 1922). The bishydroxycoumarin responsible for the disorder was formed from coumarin by fungal fermentation (Schofield 1924, Campbell and Link 1941). Already in the 1940´s, bishydroxycoumarin was synthesized and used as an oral anti-coagulant. Another area in medicine utilising coumarin derivatives is the treatment of psoriasis and other skin diseases, such as cutaneous T-cell lymphoma, atopic dermatitis, *alopecia areata*, *urticaria pigmentosa*, and *lichen planus* (Oliver and Winkelmann 1993, Goodman and Gilman 2006) with linear furanocoumarins *i.e.* psoralens and ultraviolet light (P-UVA). The most used compound is xanthotoxin (Conconi *et al.* 1998), but bergapten has also been successfully used for the treatment of psoriasis and vitiligo (McNeely and Goa 1998). The bioactivities reported for the coumarins present in *A. archangelica* and *P. palustre* can be approximately divided into six categories: anti-inflammatory, anti-microbial, anti-cancer, calcium antagonistic, effects on central nervous system, and other bioactivities.

Anti-inflammatory activity: Mammalian cells express a constitutive enzyme (COX-1) responsible for the production of prostaglandins, and an inducible isoform (COX-2) induced in response to proinflammatory cytokines and bacterial cell components (Payá *et al.* 1997). COX-1 is mainly associated with homeostasis, and COX-2 with oedematous,

nociceptive, and pyretic effects of inflammation. The nitric oxide (NO), synthesized by nitric oxide synthase (NOS), is an important mediator in inflammation (Dugas *et al*. 1995). The products of 5-lipoxygenase (5-LO) contribute to inflammation. These enzymes are essential components of the inflammatory response mechanism and are involved in the pathogenesis of several inflammatory diseases (Vane *et al.* 1994).

Columbianadin, bergapten, and umbelliferone show significant anti-inflammatory and analgesic activity. However, osthol and xanthotoxin only have anti-inflammatory activity and isoimperatorin only analgesic effect. The anti-inflammatory and analgesic constituents seem to be related to the peripheral inhibition of inflammatory substances and to their effect on the central nervous system (Chen *et al.* 1995). Lino *et al.* (1997) showed that the anti-nociseptive activity of coumarin and umbelliferone did not involve the opioid system because pre-treatment with naloxone, a competitive μ-opioid receptor antagonist, did not reverse the anti-nociception. Pre-treatment with L-arginine, which is a substrate for NO synthesis, reversed the anti-nociception caused by umbelliferone, indicating the involvement of NO. Coumarin and umbelliferone also had a significant anti-oedemic effect in the carrageenan model, but only coumarin decreased the rat paw volume in the dextran model in mice. Testing of the anti-inflammatory properties of psoralen and imperatorin in mice suggested that the anti-inflammatory activity of each compound depends on its individual substitution on the aromatic ring rather than on the coumarin skeleton itself (García-Argáez 2000). Osthol and osthenol showed an inhibitory effect on 5-LO and COX-1 *in vitro* (Liu *et al.* 1998). Also Resch *et al.* (1998) found osthol to be a moderate but selective 5-LO inhibitor. Imperatorin and isoimperatorin showed dual inhibitory activity due to their significant effect on 5-LO and comparable inhibition on COX-1- and COX-2, when compared to indometacin and nimesulide. Only imperatorin caused a significant reduction of NO generation (Abad *et al.* 2001). Also psoralen, xanthotoxin, and umbelliferone have shown COX-2/5-LO dual inhibitory activity (Kim *et al.* 2006). In contrast to the results obtained in the study of Chen *et al.* (1995), osthol was only slightly active in 5-LO inhibition but inactive in COX-1 inhibition (Roos *et al.* 1997). Of the coumarins isolated from *Angelica archangelica,* no marked inhibitory activity in the COX-1 study was found except for angelicin. In the 5-LO test, apart from osthol, only oxypeucedanin hydrate isovalerate showed linear, concentration-dependent inhibitory activity. Phellopterin, imperatorin, and archangelicin were weakly active, but had no linear concentration-dependent activity, which might be due to the calcium antagonistic properties of the coumarins as reported by Härmälä *et al.* (1992B).

Calcium antagonistic activity: The effects of columbianadin, oxypeucedanin, isoimperatorin, and ostruthol from *P. palustre* on prolactin (PRL) release from GH_3 rat pituitary tumour cells were studied using verapamil as a reference. Columbianadin inhibited the basal and thyrotropin releasing hormone (TRH)-induced PRL release, oxypeucedanin was active against KCl-induced PRL release, ostruthol showed activity at a concentration of 10^{-4} M in all cases, and isoimperatorin was active against the basal and KCI induced PRL release (Vuorela *et al.* 1988). In more detailed studies, columbianadin was found to inhibit depolarization induced Ca^{2+} uptake in rat pituitary GH_3 cells (Törnquist and Vuorela 1990). 2'-angeloyl-3'-isovalerylvaginate, archangelicin, oxypeucedanin hydrate, bergapten, byakangelicin angelate, imperatorin, isoimperatorin, isopimpinellin, 8-[2-(3-methylbutyroxy)-3-hydroxy-3-methylbutyroxy]psoralen, osthol, ostruthol, oxypeucedanin, phellopterin, psoralen, and xanthotoxin from the roots of *A. archangelica* L. subsp. *archangelica* all exhibited calcium antagonistic activity on the uptake of $^{45}Ca^{2+}$ in clonal rat pituitary GH_4C_1 cells, archangelicin being even more active than verapamil (Härmälä *et al.* 1992B). The studies carried out by Kummala and co-workers (1996) on ATP- and thapsigargin-induced changes in intracellular free Ca^{2+} in Fluo-3-AM loaded rat thyroid FRTL-5 cells, also indicated that osthol and columbianadin are potential calcium antagonists. Ojala *et al.* (2001) showed further that osthol modulates TRH-evoked responses by interacting with the TRH receptor.

Anti-microbial activity: Psoralen, 8-methoxypsoralen, bergapten, oxypeucedanin, and isopimpinellin from parsley leaves inhibited a DNA repair-deficient *Escherichia coli*, the human pathogens *E. coli* and *Listeria monocytogenes*, the spoilage micro-organism *Erwinia carotovora*, and *Listeria innocua,* but not *Pseudomonas fragi*. Photoactivation of the furanocoumarins with UV light at 365 nm for 60 min had only a marginal effect on *L. monocytogenes* and *L. innocua,* but a slight UV inhibitory effect was detected with *E. carotovora* (Manderfeld *et al.* 1997).

Kofinas *et al.* (1998) showed that umbelliferone, isopimpinellin, xanthotoxin, isobergapten, columbianetin acetate, and columbianetin were active against *Cladosporium cucumerinum*. A group of coumarins was synthesised using angelicin as the lead structure. The synthesized coumarins and angelicin derivatives were tested against *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cereviciae,* and *Aspergillus niger*. In many of the synthesised coumarins and angular furanocoumarins, the free 6-OH was found to be important for anti-fungal activity, and the free 7-OH of the coumarin nucleus for anti-

bacterial activity. Angelicin showed no cytotoxicity against Human cell line KB cells. (Sardari *et al.* 1999).

The crude extracts from the fruits of *Angelica lucida* and the isolated imperatorin, isoimperatorin, heraclenol, oxypeucedanin hydrate, and heraclenin, were moderately active against Gram positive and Gram negative bacteria and oral pathogens, while the tested extracts and isolated compounds were inactive against the assayed *Candida* species. An isoprenyl unit attached to the carbocyclic ring was found to favour the anti-microbial activity. A prenyl group in the furanocoumarin skeleton results in an increase in lipophilicity, thereby facilitating passage though the bacterial membrane (Widelski *et al.* 2009).

Umbelliprenin, imperatorin, bergapten, isopimpinellin, byakangelicin, 7-methoxycoumarin, and 5-hydroxy-8-methoxypsoralen were isolated from seeds of *Peucedanum zenkeri* L., of which only imperatorin, bergapten, and isopimpinellin had anti-microbial activity (Ngwendson *et al.* 2003). Coumarins and structurally related compounds, including imperatorin, were active against anti-human immunodeficiency virus, type 1 (HIV-1), highlighting the potential of the Sp1 transcription factor as a target for natural anti-HIV-1 compounds such as furanocoumarins (Sancho *et al.* 2004).

Anti-cancer effects: The coumarin fractions from the fruits of *Archangelica officinalis* Hoffm. and *Pastinaca sativa* L. inhibited the growth of a culture of cancer cells HeLa-S 3 grown in the dark. Ten coumarins were isolated from the fractions in decreasing order of activity, osthol, xanthotoxol, 4-methylaesculetin, isopimpinellin, bergapten, xanthotoxin, imperatorin, coumarin, umbelliferone, and 4-hydroxycoumarin, of which eight inhibited the proliferation of HeLa cells (Gawron and Głowniak 1987).

Psoralen, bergapten, and xanthotoxin from *Angelica keiskei* Koidz showed anti-tumour activity. Imperatorin and isoimperatorin showed potent inhibitory activity in a test based on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated ^{32}Pi incorporation into the phospholipids of cultured cells (Okuyama *et al.* 1990). Okuyama *et al.* (1991) also isolated the angular furanocoumarins, archangelicin and 8(*S*),9(*R*)-9-angeloyloxy-8,9-dihydrooroselol, and the linear furanocoumarins, psoralen, bergapten, and xanthotoxin. The angular furanocoumarins, suppressed (TPA)-stimulated ^{32}Pi-incorporation into the phospholipids of cultured cells, whereas the other coumarins were less active.

A chloroform extract of *Angelica japonica* root inhibited cell growth of human gastric adenocarcinoma (MK-1). The furanocoumarins osthol, isoimperatorin, scopoletin, byakangelicin, xanthotoxin, bergapten, oxypeucedanin methanolate, and oxypeucedanin hydrate, isolated from the extract, showed only weak anti-proliferative activity against MK-1,

HeLa, and B16F10 cell lines, although a number of compounds did have some specificity. Scopoletin, japoangelone, and oxypeucedanin methanolate showed higher activity to B16F10 cells than to MK-1 and HeLa cells, whereas xanthotoxin and bergapten were more active to HeLa cells than to MK-1 and B16F10 cells (Fujioka *et al.* 1999).

A total of 33 coumarins, simple isopentenylated coumarins, pyrano- and furanocoumarin derivatives, with only weak anti-proliferative activity against the normal human cell lines, showed anti-proliferative activity against cancer cell lines. The decreasing rank order of potency was osthenone, clausarin, clausenidin, dentatin, nordentatin, imperatorin, seselin, xanthyletin, suberosin, phebalosin, and osthol. The structure-activity relationship established on the basis of the results showed that the 1,1-dimethylallyl and isopentenyl groups play an important role in anti-proliferative activity (Kawaii *et al.* 2001).

Kleiner *et al.* (2001) showed that oral administration of coumarins leads to altered enzyme activities and reduced DNA adduct formation by polycyclic aromatic hydrocarbons in the tissues of SENCAR mice. Imperatorin and isopimpinellin significantly blocked the cytochrome P-450 enzymes ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-dealkylase (PROD) in epidermis. They also modestly inhibited EROD activities in the lung and forestomach, and significantly inhibited PROD activities in the lung and forestomach. After 24 h of the final oral dose of imperatorin or isopimpinellin, EROD and PROD activities remained inhibited in the epidermis and lung. However, forestomach P-450 activity returned to the control levels. They also increased by 1.6-fold liver cytosolic glutathione *S*-transferase activity at both 1 and 24 h after the final oral dose compared with the controls. Oral administration also provided protection against DNA adduct formation by benzo[*a*]pyrene (B[*a*]P) and 7,12-dimethylbenzyl[*a*]anthracene (DMBA). Imperatorin pre-treatment decreased the formation of DNA adducts by (DMBA) in the forestomach. Pre-treatment with isopimpinellin led to reduced DNA adduct levels in liver (B[*a*]P), lung (B[*a*]P), and mammary epithelial cells (DMBA), suggesting that imperatorin and isopimpinellin may have potential chemo-preventive effects. Imperatorin induced apoptosis in human promyelocytic leukaemia, HL-60 cells. Neither necrosis nor differentiation was observed at cytotoxic micromolar concentrations (Pae *et al.* 2002).

Effects on the central nervous system: Coumarins have shown activity in several receptor systems of the central nervous system. Phellopterin strongly inhibited the binding of [^{3}H]diazepam to benzodiazepine receptors *in vitro*, while the other coumarins, despite their structural similarities, were considerably less active (Bergendorff *et al.* 1997). Huong *et al.* (1999) reported that xanthotoxin, psoralen, and bergapten inhibited monoamino oxidase

enzyme (MAO) *in vitro*. Imperatorin has been shown to elevate the gamma-aminobutyric acid (GABA) levels in both a time- and concentration-dependent manner by inhibiting the GABA degradative enzyme gamma-aminobutyric acid transaminase GABA-T. However, isoimperatorin, phellopterin, and oxypeucedanin hydrate were inactive both in GABA-T and succinic semialdehyde dehydrogenase tests (Choi *et al.* 2005). Imperatorin has also been reported to exhibit affinity towards the 5-HT_7 receptors in a competitive binding assay (Deng *et al.* 2006). Ostruthol, imperatorin, isoimperatorin, and oxypeucedanin hydrate inhibited acetylcholine esterase (AChE), being about ten-fold more active than the AChE inhibitor galanthamine, and almost as strong as huperzine A from *Huperzia serrata*, Lycopodiaceae, which is presently the most powerful known AChE inhibitor (Hostettmann and Marston 2007).

Other bioactivities: Oxypeucedanin, bergapten, xanthotoxin, imperatorin, and phellopterin activated adrenaline-induced lipolysis in fat cells from rats. Oxypeucedanin hydrate, imperatorin, and phellopterin also activated adrenocorticotropin-induced lipolysis. In contrast, furanocoumarins such as byakangelicin, neobyakangelicol, and isopimpinellin strongly inhibited insulin-stimulated lipogenesis (Kimura *et al.* 1982). A diet containing 0.05% osthol over 4 weeks in 6-week-old, male, stroke-prone, spontaneously hypertensive rats significantly suppressed the elevation of systolic blood pressure and decreased cholesterol and triglyceride contents in the liver without any significant changes in serum lipids (Ogawa *et al.* 2007). Among the isolates from *Peucedanum japonicum,* eugenin, (-)-selinidin, (+)-pteryxin, imperatorin, bergapten, cnidilin, and (+)-visamminol, showed strong anti-platelet aggregation activity *in vitro* (Chen *et al.* 1996). Osthol, imperatorin, xanthotoxin, and isopimpinellin showed a relaxing effect in the phenylephrine-precontracted endothelium of intact rabbit *corpus cavernosum* (Chiou *et al.* 2001).

Eleven furanocoumarins expressed estrogenic activity on the Ishikawa cell line. 9-hydroxy-4-methoxypsoralen and alloisoimperatorin were the most active, whereas oxypeucedanin hydrate, 9-hydroxy-4-methoxypsoralen, byakangelicin, pabulenol, alloisoimperatorin, neobyakangelicol, byakangelicol, oxypeucedanin, imperatorin, phellotorin, and isoimperatorin were only slightly active (Piao *et al.* 2006).

6.5 The role of coumarins in plants

The primary site of synthesis of coumarins is suggested to be the young, actively growing leaves, with other organs playing a comparatively minor role (Murray *et al.* 1982).

However, this may vary between species and compounds, *e.g.* furanocoumarins in *Pastinaca sativa* are formed in the fruits (Zangerl *et al.* 1989), and furanocoumarins in *A. archangelica* are formed in the leaves with the exception of osthenol, a simple coumarin, which is probably formed in the roots (Murray *et al.* 1982). Apiaceae plants are known to accumulate coumarins in oil tubes of the fruit and in the seed coats, as reported in *Pastinaca sativa* (Zangerl *et al.* 1989). In *A. archangelica* and *Heracleum lanatum,* high levels of coumarins were found in the seeds but low levels in the fruit tissues (Zobel and Brown 1991), whereas the seeds of the Rutaceae had much smaller concentrations of furanocoumarins both on the surface and within the seed. Coumarins are also found in the green tissue (*e.g.* Vuorela 1988, Hadaček 1989). Many plants excrete their coumarins on the leaf surface (Zobel and Brown 1990A, Zobel *et al.* 1991B). This was monitored by selective extraction of the leaf surface (Zobel and Brown 1988) in *Heracleum lanatum, Ferula communis, var. glauca, Pastinaca sativa, Apium graveolens, Pimpinella anisum, Psoralea bituminosa* Leguminosae, and *Citrus limon* Rutaceae. The higher concentration generally observed in spring leaves compared to autumn leaves suggests a higher rate of transfer of these furanocoumarins to the surface in younger leaves. Accumulation on the plant surface indicates they play a role in the defence mechanism of the plant (Zobel and Brown 1990B).

The bergapten level in the petiole and leaf tissue of *Apium graveolens* increased during development and declined only in the later stages of maturity and showed a clear seasonal trend (Trumble *et al.* 1992). Lois and Hahlbrock (1992) reported that the older leaves of parsley contained more furanocoumarin-specific bergaptol-*O*-methyltransferases, which may explain this phenomenon. In *Heracleum lanatum* the amount of coumarins increased up until mid-May, and decreased thereafter until maturity. In the small autumn leaves the coumarin concentration was 2-3 times higher than that in April. A similar analysis of mature *Ruta graveolens* plants (Zobel and Brown 1990B) revealed that the proportion of bergapten in comparison to psoralen and xanthotoxin increased during senescence. In embryogenic cultures of *P. palustre* the amount of columbianadin and oxypeucedanin increased along with the development of embryos, and in regenerated plants the oxypeucedanin content was the highest in young plants but decreased significantly in 3-month old plants (Härmälä *et al.* 1992A). In intact plants of *P. palustre* the coumarin content is the highest in the beginning of the growing season, then decreases for approximately 2 weeks, but remains constant during the following 4 weeks. Towards the end of the growing season the coumarin content again begins to increase (Kummala *et al.* 1993).

Exposure to air pollution-simulating acid fog comprising HNO_3 and H_2SO_4 at pH 2.0 for 4 h increased the coumarin content of the leaves and petioles of *Apium graveolens* L. approximately fivefold at 120 h after the start of the treatment (Dercks *et al.* 1990). In the same report, single ozone fumigation at 0.20 ppm for 2 h generally reduced the concentrations of furanocoumarin in the leaves of celery within 24 h, but the levels of these compounds then increased rapidly and the concentrations did not differ significantly at 120 h. Unlike the case with *A. graveolens* L., spraying *Ruta graveolens* plants with H_2SO_4 at pH 2.4 or saturated NaCl solutions decreased the total concentration of furanocoumarins (Zobel *et al.* 1991A). In *Petroselinum crispum* L. a single ozone treatment (200 nL L^{-1}) for l2 h induced both flavonoid glucoside and furanocoumarin biosynthesis, the furanocoumarin concentration being the highest at 12h and 24 h after initiation of the exposure (Eckey-Kaltenbach *et al.* 1994). Exposure to 1 mM $CuCl_2$ and irradiation with short wavelength UV light lead to the accumulation of scopoletin and ayapin in *Helianthus annuus* (Gutierrez *et al.* 1995), while long wave UV irradiation had no effect. Celery exposed to $CuSO_4$, UV light, and cold produced psoralen, bergapten, xanthotoxin, and isopimpinellin. The phytoalexin distribution was found to be temperature dependent. Studies with $CuSO_4$ exposure showed that the onset of the response was also concentration dependent (Beier and Oertli 1983). In the study of Trumble *et al.* (1992), pesticides showed no marked effect on furanocoumarin formation in *Apium graveolens* L., but Nigg *et al.* (1997) reported that fungicide treatment of a commercial celery cultivar increased bergapten 2-4 times in the leaves and stalk, xanthotoxin 2-3 times in the stalk, and isopimpinellin 2-3 times in the leaves. However, the treatment did not increase the psoralen levels. Coumarins are also active in plant metabolism, taking part in growth regulation (Weinmann, 1997, Matern *et al.* 1999), and their excretion on the surface of seeds might be a means to delay germination (Zobel and Brown 1991). This could also be the reason for poor germination ability of some species (Ojala 1985).

The distribution of biologically active coumarins in a wide range of plants seems to not only correlate with their ability to act as a response to injury, wilting, chemical or environmental stress factors, but they also act as a defence mechanism against plant diseases or insects (Weinmann, 1997, Matern *et al.* 1999). Coxon *et al.* (1973) showed that scopoletin was formed as a phytoalexin in carrots exposed to ethylene and various fungi. Johnson *et al.* (1973) showed that the inoculation of parsnip root disks with the fungi *Ceratocystis fimbriata*, *Helminthosporium carbonum*, *Alternaria* species or *Colletotricum lindemuthianum*, which are non-pathogens of parsnip, resulted in the accumulation of xanthotoxin which also inhibited the growth of *C. fimbriata*. *A. archangelica* root discs

infected *in vitro* with *Aspergillus niger, Fomes fomentarius, Heterobasidion annosum, Inonotus obliquus,* and *Phellinus igniarius* were found to contain increased levels of bergapten, imperatorin, ostruthol, oxypeucedanin, and xanthotoxin. Changes in the concentration of these furanocoumarins were dependent on the time of infection and on the fungus species (Głowniak *et al.* 1994).

Ojala *et al.* (2000) studied the toxicity of plant extracts from *Aegopodium podagraria* L., *Anethum graveolen*s L., *Angelica archangelica* L., *Levisticum officinale* Koch, *Petroselinum crispum* (P. Mill.) A.W. Hill., *P. palustre* (L.) Moench, and *Ruta graveolens* L. against Gram-positive and Gram-negative bacteria, yeasts, mould, and plant pathogenic fungi. *Petroselinum crispum* and *Ruta graveolens* extracts showed the highest toxicity against *Rhizoctonia solani*. The growth of *Heterobasidium annosum* was inhibited, whereas that of *Phytophtora* (*cactorum*) was actually promoted. The anti-bacterial and anti-fungal activities of bergapten, coumarin, herniarin, umbelliferone, xanthotoxin, and scopoletin were weak, except for the inhibitory effect against *Fusarium culmorum*. Inoculation with a spore suspension of the fungus *Helminthosporium turcicum,* as well as with aqueous solutions of mercuric chloride and cupric chloride, lead to the production of scopoletin, fraxinol, isopimpinellin, xanthotoxol, and peucedanol in the fresh young leaves of *Corchorus olitorius* L. (Zeid 2002). Coumarins usually possess only a weak to moderate anti-microbial activity. In defence against micro-organisms other compounds may, however, potentiate the effect of coumarins. Scopoletin was isolated together with vanillin, 4-hydroxy-3-methoxycinnamaldehyde, and ±-pinoresinol from the seed kernels of *Melia azedarach* L.. Scopoletin alone showed weak activity but, when combined with the above-mentioned compounds, strong enhancement of the anti-fungal effect was observed (Carpinella *et al.* 2005).

Virus infections may also stimulate coumarin production. Celery infected with celery mosaic virus or parsnip yellow fleck virus contained angelicin, bergapten, psoralen, trimethylpsoralen, and xanthotoxin, but were not detected in celery plants grown in aseptic culture. They are found only occasionally in apparently healthy plants in the glasshouse (Lord *et al.* 1988).

In order to assess the degree of genetic control over induction, levels of genetic variation, and genetic correlation, induced and constitutive furanocoumarins were measured in two populations of *Pastinaca sativa*. Artificially damaged leaflets of *P. sativa,* as well as undamaged reference leaflets, were analyzed for furanocoumarins. The same plants were used to determine the frequency of attack by leaf-feeding herbivores. Both populations

experienced similar frequencies of attack, but the overall infestation rate varied. The two populations contained significant amounts of genetic variation for constitutive and induced levels of furanocoumarins, and differed significantly in the constitutive levels of two of the furanocoumarins. The population that experienced a low probability of herbivory contained significantly less furanocoumarin. The populations had differing defence strategies that were in accordance with the probability of being attacked, thereby showing that selection can act on the inducibility of plant chemical defences (Zangerl and Berenbaum 1990).

In the interaction between the parsnip webworm larvae (*Depressaria pastinacella*, de Geer), and *Pastinaca sativa,* traits for plant resistance to insect herbivory through the production of defensive furanocoumarins, as well as traits for herbivore virulence through the ability to metabolize furanocoumarins are characterized by continuous heritable variation. The *P. sativa* traits were classified into four clusters describing multi-trait phenotypes occurring in all or most of the populations. When the frequency of plant phenotypes belonging to each of the clusters was compared with the frequency of the insect phenotypes in each of the clusters across the populations, a considerable degree of frequency matching was revealed in 3 of the populations. Such differences among populations may result from cycling caused by frequency-dependent selection. Changes in phenotype frequencies may occur when the plant population develops in order to reduce the level of interaction with the insects, and the insects then develop to increase the level of interaction (Berenbaum and Zangerl 1998).

Hadaček *et al.* (1994) studied the insecticidal activity of 17 coumarins present in *Peucedanum arenarium*, *P. austriacum*, *P. coriaceum*, *P. longifolium*, *P. officinale* L., *P. oreoselinum*, *P. ostruthium* L., and *P. palustre* against *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). The majority of the linear furanocoumarins and the angular dihydrofuranocoumarin athamantin were active. Oxygenation of the prenyl residue of linear furanocoumarins decreased the activity and further esterification with angelic acid lead to inactivity. Active linear furanocoumarins, bergapten, isopimpinellin, and imperatorin, and linear furanocoumarins with a substituted furan ring, peucedanin and 8-methoxypeucedanin, were compared in a dietary utilization bioassay. The relative growth rate (RGR) and relative consumption rate (RCR) divided the coumarins into three groups. Isopimpinellin and peucedanin slightly decreased RGR and RCR of the larvae, and xanthotoxin, isoimperatorin, and 8-methoxypeucedanin strongly decreased RGR and RCR. Bergapten and imperatorin differed in having the lowest RGR values and relatively high RCR values, thereby indicating

specific postingestive toxicity. Thus, coumarins are effective in chemical defence, when we assume that chemical diversity is a necessary trait for well-defended plants.

Exposure of leaves of *Apium graveolens* to vapours of methyl jasmonate (MeJa), or feeding the petiole with an aqueous solution of jasmonic acid (JA), markedly increased the levels of natural furanocoumarins, in particular of xanthotoxin and bergapten. The levels of xanthotoxin and bergapten started to increase approx. 24 h after induction, reaching the maximum concentration after 4-6 days (Miksch and Boland 1996). Ellard-Ivey and Douglas (1996) used *Petroselinum crispum* cell cultures and transgenic *Nicotiana tabacum* plants expressing the 4CL1-GUS gene. Jasmonates and α-linolenic acid strongly induced the expression of 4CL dose-dependently in parsley cells. MeJa also activated the co-ordinate expression of other phenylpropanoid genes and the accumulation of furanocoumarins. In transgenic wound-inducible tobacco, 4CL genes and a 4CL1 promoter-GUS transgene were dose-dependently responsive to jasmonates and α-linolenic acid. Pre-treatment with a lipoxygenase inhibitor reduced their responsiveness to the elicitor and to wounding, showing that elicitation can be partially mimicked by jasmonate treatment, thereby supporting the role of jasmonates in mediating wound-induced expression of 4CL and other phenylpropanoid genes.

Treatment of *Apium graveolens*, cv. *secalinum* leaves with JA or analogues of amino-acid conjugates of JA such as the leucine conjugate 1-oxoindane-4-carboxylic acid stimulated the biosynthesis of the furanocoumarins psoralen, xanthotoxin, bergapten, and isopimpinellin. In addition to the increase in coumarins within the leaf, a significant amount (20%) of the total furanocoumarins was deposited on the surface of the leaf. Xanthotoxin and bergapten began to increase steadily and simultaneously within the leaf and on the leaf surface 40 h after JA stimulation. Females of the carrot fly (*Chamaepsila rosae*, Psilidae) responded with an increased oviposition to the altered leaf surface chemistry, *i.e.* to the elevated amounts of furanocoumarins (Stanjek *at al.* 1997). Quite the opposite was reported by Black and co-workers in (2003) with leafminers (*Diptera*: Agromyzidae), who demonstrated that conventionally grown celery had more leafminer oviposition stings than the plants treated with JA or untreated control plants, suggesting that in certain cases JA elicitated coumarin induction could be used as an alternative pest repellent.

Transgenic *Arabidopsis thaliana* overexpressing JA-carboxyl-methyltransferase (JMT) had a 3-fold elevated level of endogenous MeJa. The expression of the gene was inducible both locally and systemically by wounding or by the MeJa treatment, suggesting that JMT can perceive and respond to local and systemic signals generated by external

stimuli, and the signals may include MeJa itself. The transgenic plants exhibited constitutive expression of jasmonate-responsive genes, and the resistance against the virulent fungus *Botrytis cinerea* was enhanced. Thus, JMT could be a key enzyme for jasmonate-regulated plant responses. Activation of JMT expression leads to the production of MeJa, which acts as an intracellular regulator, an intercellular signal transducer, and an airborne signal in intra- and interplant communications (Seo *et al.* 2001).

Wen *et al.* (2006) studied the effects of allelochemicals in host plants of the black swallowtail *Papilio polyxenes* on the xanthotoxin-metabolic activity of CYP6B1, the enzyme responsible for the detoxification of furanocoumarins. The integrity of furanocoumarin structure was shown to be important for competitive binding to the active site of CYP6B1, even though the carbonyl group on the pyranone ring apparently did not affect its inhibitory capacity. Angular furanocoumarins are generally less phototoxic than linear furanocoumarins, but more toxic than linear furanocoumarins, to black swallowtail larvae. This enhanced toxicity *in vivo* may be due to the ability of angular furanocoumarins to bind to the active site of CYP6B1, thereby reducing the availability of CYP6B1 to metabolize other linear furanocoumarins.

6.6 Somatic embryogenesis

The Apiaceae family is known to possess embryogenic ability. Somatic embryogenesis has been reported in over 30 Apiaceae species (see **Table 3**). In theory, all healthy plant cells are totipotent. Plants are thus able to produce morphologically and developmentally normal embryos from undifferentiated somatic cells. This is called somatic embryogenesis, which is the opposite to zygotic embryogenesis resulting from the fertilization of an egg cell. Somatic embryogenesis can be divided into four phases: induction, growth, maturation, and germination (Ammirato 1983). Embryogenesis can start directly from a cell or tissue without previous callus formation, or indirectly with callus formation from which the embryos are subsequently formed. Embryos at the first recognizable stage are globular as they grow in a small cluster of cells. After that they start growing isodiametrically, with heart- and torpedo-shaped stages following (Dudits *et al.* 1991). Characteristic of the globular-to-heart transition is the outgrowth of the two cotyledons, elongation of the hypocotyls, and the start of root development. This is followed by the torpedo stage up to the plantlet. Plantlets can then be transplanted into solid medium for the regeneration of whole plants (Pierik 1987).

Somatic embryogenesis was first described in the international scientific literature in callus and suspension cultures of *Daucus carota* L. (Reinert 1958, Steward *et al.* 1958). Interestingly, Waris in his report on seedlings of *Oenanthe aquatica* L., maintained in culture for long periods without exogenous growth hormones, described a phenomenon that is clearly somatic embryogenesis already in 1957. Thus it is clear that Waris was amongst the very first to observe and report somatic embryogenesis (Waris 1957, Krikorian and Simola 1999). According to Ammirato (1983), it was already possible in 1979 to induce somatic embryogenesis in 132 plant species that are fairly common in the Ranunculaceae, Rutaceae, Solanaceae, Umbelliferae and Gramineae families (Tisserat *et al.* 1979).

Carbohydrates. In the great majority of the reports, standard growing media such as Murashige and Skoog (1962) or Gamborg (1968) media have been utilised with minor modifications, the most commonly used carbohydrate thus being sucrose. Other carbohydrates have also been tested either as carbon sources or as osmotic regulators. Glucose, fructose, galactose, mannose, maltose, raffinose, and stachyose are suitable carbon sources for *Daucus carota* L. embryogenic suspension cultures (Verma and Dougall 1977). Chopra and Khanna reported (1979) that sugar was essential for the rhizogenesis of *Anethum graveolens* embryos. Nadel *et al.* (1989) showed that, in embryogenic suspension cultures of *Apium graveolens,* the addition of mannitol prevented cell lysis, increased the number of singular somatic embryos, improved their normal differentiation, and accelerated torpedo embryo development. Experiments designed to reveal the nature of the mannitol effect have demonstrated that the decreased osmotic potential was an important factor, but that isomolar solutions of sucrose alone were not as effective. Huang *et al.* (1993) replaced sucrose with glucose in their fed-batch studies with *Daucus carota* cultures.

Nitrogen source. The composition of the nutrient medium is critical, and attention has focused on the sources and levels of nitrogen, especially of reduced nitrogen. The benefits of reduced nitrogen, in addition to nitrate, for both embryo initiation and maturation are well established. Reduced nitrogen can be supplied as ammonium ions, complex addenda such as casein hydrolysate, or as amino acids. Smith and Krikorian (1989) studied carrot *Daucus carota* on media containing unreduced nitrogen. Somatic embryo formation led to the generation of vigorous cultures comprising entirely of somatic embryos at various stages of development, which, in turn, further proliferated other somatic embryos. However, media with 1-5 mM NH_4^+ as the sole nitrogen source, led to a proliferation of globular proembryos. Sustained sub-culturing of these proembryos at two to three week intervals enabled establishment of highly uniform cultures in which no further development into more mature

stages of embryonic development occurred. A basal medium containing 1-5 mM NH_4^+ as the sole nitrogen source does not appear to be inductive to somatic proembryo formation. Instead, such a medium is best considered to be permissive to the expression of embryogenically determined cells within zygotic embryos.

Niedz (1994) cultured embryogenic callus from *Citrus sinensis* (L.) in 20 media arranged in a 5 x 2 x 2 factorial, with a varying ratio of NO_3^- to NH_4^+ nitrogen, total inorganic nitrogen, and benzyladenine. The fresh weight increase in callus and final pH of the medium were significantly affected by total inorganic nitrogen and the ratio of NO_3^- and NH_4^+. The NO_3^-/NH_4^+ -ratio accounted for 55% of the variation in the fresh weight increase in callus, and 93% of the variation in the final pH of the medium. Varying the NO_3^-/NH_4^+ -ratio provided adequate pH control.

Barrett *et al.* (1997) investigated the effects of the glutamine-based dipeptides, glutamine, and casein hydrolysate, as well as the deletion of organic nitrogen, during somatic embryogenesis of *Picea glauca* (Moench) Voss. There were no differences in the increase in the fresh weight of the tissue mass grown on initiation medium with different combinations of organic nitrogen. This was also the case for subsequent growth on kinetin medium, except that glutamine alone produced a significantly lower fresh weight increase than the other organic nitrogen combinations. With only inorganic nitrogen in the medium, the fresh weight increase was significantly less than that with organic nitrogen in both the initiation and kinetin medium. No differences were found between the DM/FM ratios among the various nitrogen treatments. The number of mature embryos when cultured in the absence of organic nitrogen was significantly higher than that obtained in its presence. There were no differences in the total number of mature embryos produced in cultures grown with various organic nitrogen combinations or without organic nitrogen, but there were large clone differences with respect to the number of mature somatic embryos per gram tissue and the total number of somatic embryos produced.

Plant growth regulators. Plant hormones play a critical role in the establishment of somatic embryogenesis. Recent studies have concentrated on the interactions between exogenously added plant growth regulators and the endogenous hormones present in the plant material. The sensitivity of the tissues to various hormones also has a marked position in understanding the phenomenon (Jiménes 2005).

Auxins and cytokinins are commonly used plant growth regulators in media in order to induce embryogenesis, but auxins usually inhibit the further development of embryos. Of all the auxin-like plant growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D) has been most

used, but other auxins such as naphtalene acetic acid (NAA), indole acetic acid (IAA), and indole butyric acid (IBA) have also been used frequently. Also IAA-amino acid conjugates have been shown to trigger somatic embryogenesis (Newton and Shea 2006). Cytokinins, as well as gibberellins and abscisic acid (ABA), play an important role in embryogenesis in a number of species (*e.g.* Ammirato 1977).

Kikuchi *et al.* (2006) found a relationship between endogenous ABA and the induction of somatic embryogenesis using the stress-induced system of somatic embryos. Somatic embryo formation was inhibited by the application of fluridone, a potent inhibitor of ABA biosynthesis, during the stress treatment. The inhibitory effect of fluridone was reversed by the concomitant application of fluridone and ABA. The level of endogenous ABA increased transiently during stress. However, somatic embryogenesis was not significantly induced by the application of only ABA to the endogenous level in the absence of stress, suggesting that the induction of somatic embryogenesis, in particular the acquisition of embryogenic competence, is caused not only by the presence of ABA but also by physiological responses that are directly controlled by stresses.

There are cases in which no plant growth regulators are needed for maintaining the embryogenicity (*e.g.* Masuda *et al.* 1977, Smith and Krikorian 1990, Vuorela *et al.* 1993) The gaseous environment also affects the initiation, proliferation, and maturation of somatic embryos (*e.g.* Jay *et al.* 1992, Huang *et al.* 2006).

Overvoorde and Grimes (1994) studied the role of Ca^{2+} and calmodulin in carrot somatic embryo formation. Embryogenic cell clumps were induced to form embryos in medium containing 0-3 mM Ca^{2+}. Embryo formation was not affected until the concentration of Ca^{2+} was lower than 200 mM. Lower concentrations decreased embryo formation. The treatment of developing embryos with Ca^{2+}-channel blockers verapamil or nifedipine, or the Ca^{2+} ionophore A23187, inhibited embryo formation, which suggests that exogenous Ca^{2+} or the maintenance of Ca^{2+} gradients is required for proper embryo development. Mashayekhi and Neumann (2006) showed that another micronutrient, boron, has a strong influence on the development of somatic embryos in the concentration range of 0 to8 mg L^{-1}. At lower boron concentrations the development of roots is promoted with simultaneous retardation of shoot development, and at higher boron concentrations shoot development is favoured over development of the root system. Parallel to this, the ratio between the concentration of endogenous indole-3-acetic acid and total cytokinins changed continuously from 4 (zero boron) to 0.22 (8 mg L^{-1} boron). An increase in the boron concentration in the nutrient medium generally resulted in a reduced concentration of endogenous abscisic acid.

Table 3: *In vitro* cultures from Apiaceae species in which embryogenesis and regenerative processes have been studied

Species	Explant	Regeneration stage	Reference
Ammi majus L.	Ovary	Somatic embryos	Sehgal 1972
	Hypocotyl	Somatic embryos	Grewal *et al.* 1976
	Cotyledonary leave	Shoots, plantlets	Purohit *et al.* 1995
Ammi visnaga (L.) Lam.	Hypocotyl callus	Somatic embryos	EI-Fiky *et al.* 1989
	Cotyledonary leave	Somatic embryos	EI-Fiky *et al.* 1989
Anethum graveolens L.	Petiole, leaf, seedling	Somatic embryos	Schäfer *et al.* 1986
Angelica acutiloba (Sieb. & Zucc.)	Pedicel	Somatic embryos, plantlets	Nakagawa *et al*. 1982
Angelica archangelica L.	Seedling, callus suspension	Somatic embryos, plantlets	**(III)**
Angelica sinensis (Oliv.) Diets.	Callus from root, leaf, petiole, cotyledon, hypercotyl	Somatic embryos, plantlets or adventitious buds	Zhang, *et al.* 1982
Angelica sinensis (Oliv.) Diets.	Immature embryo	Somatic embryos, plantlets, plants	Tsay and Huang 1998,
Apium graveolens L.	Petiole callus, suspension	Somatic embryos, plantlets, plants	Williams and Collin 1976
Apium graveolens L.	Petiole callus, suspension	Somatic embryos, plantlets, plants	Nadel *et al.* 1989
Bupleurum falcatum L.	Leaf callus	Somatic embryos, plantlets, plants	Hiraoka *et al*. 1986
Carum carvi L.	Petiole	Somatic embryos	Ammirato 1977
	Hypocotyl	Somatic embryos, plantlets, plants	Furmanowa *et al*. 1984
	Petiole, leaf, seedling	Somatic embryos	Schäfer *et al*. 1986
Coriandrum sativum L.	Petiole, leaf, seedling	Somatic embryos	Schäfer *et al.* 1986
	Hypocotyl segment, zygotic embryo	Somatic embryos, plantlets, plants	Kim *et al.* 1996
	Cotyledon, hypocotyls segments	Somatic embryos, plantlets, plants	Murthy *et al.* 2008
Cuminum cyminum L.	Hypocotyl, primary leaf explant	Embryos, plantlets	Tawfik and Noga 2002
Daucus carota L	Taproot derived callus	Embryos, plantlets	Steward *et al.* 1958
Daucus carota L	Taproot derived callus	Embryos, plantlets	Reinert *et al.* 1958
Eryngium foetidum L	Root, stem disc, leaf, scape explants	Plantlets	Martin 2004
Ferula assa-foetida L.	Hypocotyl explants	Plantlets	Hassani *et al.* 2008
Foeniculum capillaceum Gilib.	Shoot tip	Shoots and plantlets	Furmanowa *et al.* 1981
Foeniculum vulgare Miller	Stem and petiole	Somatic embryos, plantlets	Hunault 1984
	Hypocotyl, stem and petiole	Somatic embryos, plantlets, plants	Reichling *et al.* 1985
Foeniculum vulgare L.	Petiole fragments		Hunault and Mataar 1995
Heracleum candicans Wall	Petiole	Somatic embryos, plantlets	Wakhlu and Sharma 1998
Oneanthe aquatica L.	Callus from seedlings	Somatic embryos, plantlets, plants	Waris 1957
Petroselinum crispum L.	Petiole, leaf and, seedling	Somatic embryos	Schäfer *et al.* 1986
Peucedanum palustre L. (Moench)	Callus, suspension	Somatic embryos, plantlets, plants	Vuorela *et al.* 1993
Pimpinella anisum L.	Hypocotyl callus, suspension	Somatic embryos	Ernst and Oesterhelt 1984
	Petiole, leaf, seedling	Somatic embryos	Schäfer *et al.* 1986
	Root explant	Somatic embryos	Bela and Shetty 1999
Thapsia garcanica L.	Suspension, callus	Somatic embryos	Jäger *et al.* 1993
Trachyspermum ammi (L.) Sprague	Hypocotyl explant	Somatic embryos , plants	Jasrai *et al.* 1992

Micropropagation. Somatic embryogenesis has been recognized as a viable means of regenerating plants from cell cultures. It offers an alternative technique for mass production (see **Table 3**). Naturally, the nature of the plant in question is a decisive factor in the economical feasibility of the process. Micropropagation is a method of vegetative plant propagation in *in vitro* culture. It guarantees, among other things, that the resulting plants are of the same genotype and phenotype. It allows the propagation of plants that cannot form seeds due to unsuitable climatic conditions or if the germination ability is poor for other reasons (Ojala 1985, Zobel and Brown 1991). What should not be forgotten is that somatic

embryogenesis may elevate the probability of mutations. The repetition of sub-culturing may also lead to the loss of regenerative capacity (Pierik 1987).

7 AIMS OF THE STUDY

The project was based on the importance of coumarins in Apiaceae family plants as possible lead compounds for drug discovery. The poor germination ability of some Apiaceae plants leads to problems in the industrial cultivation of these plants. As a result, alternative propagation methods are needed.

The main goal of the project was to create an overall protocol for coumarin analytics. The goal was also to maximise coumarin production, to optimise plant cell production, and to investigate the micropropagation possibilities offered by embryogenesis.

The aims of the study were:

- to develop analytical methods for fast and reliable screening of coumarins, the main secondary metabolites of the *Peucedanum* and *Angelica* plants **(I)**.
- to investigate the chemotaxonomic characteristics of the Finnish *Peucedanum palustre* populations, with the focus on coumarin production in order to find the best lines for the future development of suitable plants for plant propagation **(II)**.
- to generate a spontaneously embryogenic cell line of *Angelica archangelica* and to examine the possibilities of *in vitro* plant propagation by means of bioreactor technology **(III)**.
- to improve the growing conditions of the *in vitro* plant cultures, with special emphasis on optimisation of the cultivation medium **(IV)**.

8 EXPERIMENTAL

A detailed description of the materials and methods can be found in the original publications **(I) - (IV)**.

8.1 Chemicals

Columbianadin and apterin were isolated and identified from *P. palustre* (L.) Moench, and imperatorin, isoimperatorin, osthol, oxypeucedanin, and phellopterin from *A. archangelica* (L.) at the Division of Pharmacognosy, Dept. of Pharmacy, University of Helsinki, Finland. Isopimpinellin and xanthotoxin were obtained from Carl Roth GmbH & Co (Karlsruhe, Germany), bergapten and umbelliferone from Fluka Chemie AG (Buchs, Switzerland), and ostruthol from Serva (Heidelberg, Germany). Jasmonic acid was from (Sigma Chemicals Go, St. Louis, USA). HPLC-quality acetonitrile (MeCN), methanol (MeOH) and tetrahydrofurane (THF) were from Rathburn Chemicals Ltd, (Walkerburn, Scotland), and formic acid Suprapur® and ammonium acetate (analytical grade) from Merck (Darmstadt, Germany).

8.2 Sample preparation

For **(I)** the root and umbel samples of *P. palustre* (L.) Moench were collected from intact plants in Kaunissaari, Finland (60° 11′ N, 25° 22′ E). *A. archangelica var. archangelica* root samples were from intact plants collected at Seipikangas, Finland (65° 41′ N, 27° 17′ E), and the regenerated leaves from the cell line described in **(III)**. In **(II)** 132 whole flowering, healthy plants of *P. palustre* of similar size and were collected from 43 sites in southern and central Finland between 59 º 35′ and 65º 29′ N and between 21º 14′ and 29º 22′ E in late July- early August, 1988. The different plant organs were separated, dried, and stored at room temperature. In **(III)** and **(IV)**, the plant material was removed from the medium by vacuum filtration and lyophilised. The plant samples were prepared by extracting 50-100 mg of dried and pulverised plant material with 2-3 mL of MeOH for 20 min. After extraction, the samples were centrifuged (1500 G) and 5 to 50 µL of the supernatant was used for HPLC analysis **(I-IV)**.

8.3 Chromatographic analysis

The column used for method development **(I)** was a RP-C18 phenomenex® Prodigy (3 µ, 100 x 4.6 mm i.d., Torrance, CA, USA). The samples were analysed using UV detection at

320 nm with a flow rate of 1mL min^{-1}. The HPLC system used in method development consisted of a Perkin Elmer Series 200 LC pump and autosampler (Norwalk, CT, USA), and a Perkin Elmer LC 235C diode array detector at the scanning wavelength of 195-365 nm. The system was controlled by means of a Digital Venturis 575 computer including the LC method optimisation programmes, Turbo Method Development® (Perkin Elmer, Norwalk, CT, USA) and DryLab® (LC Resources, Walnut Creek, CA, USA). The MS facility for identification was a PE Sciex API 300 LC/MS/MS triple quadrupole system (Sciex, Toronto, Canada) with a heated nebulizer atmospheric pressure chemical ionisation (APCI) interface, and two Series 200 Micro LC pumps. Simultaneous UV chromatogram data were detected by a PE 785A UV/VIS detector connected to the computer by means of a PE Nelson 900 Series Interface (Norwalk, CT, USA). The MS and UV data were handled and the whole apparatus controlled by a Macintosh Power PC computer (Apple Computer Inc., Cork City, Ireland).

In **(II)** the coumarins were analysed by HPLC according to Vuorela *et al.* (1989) using isocratic runs with UV detection at 320 nm with a flow rate of 1mL min^{-1}. Identification of the coumarins was confirmed with the reference from **(I)**. The HPLC system consisted of a Waters 660 System controller, HPLC Pump 510 and HPLC Pump 6000 A (Waters Associates, Milford, MA, USA) and a Philips LC 871 UV/VIS -detector (Pye Unicam Ltd, Cambridge, England), coupled to a Hewlett-Packard Integrator plotter 3390A (Pennsylvania, USA). The column used was μBondapak C-18, 10μm, 300 × 7.8 mm i.d. (Waters Associates, Milford, MA, USA).

In **(III)** and **(IV)** the coumarin determinations were carried out by the HPLC system consisting of a Waters 600E system controller, multisolvent delivery system, Waters 717 autosampler, photodiode array detector 991 (Waters Corporation, Milford, USA), and a C-18 LiChroCART® 250-4 Hypersil ODS (5 μm) column (Merck KGaA, Darmstadt, Germany). Identification of the coumarins in the regenerated plants was based on standard compounds, retention times in HPLC, and UV and mass spectra.

8.4 Plant cell cultures in the growing experiments (III)

The embryogenic cell line of *A. archangelica* (L.) subsp. *archangelica* was established from surface-sterilized, peeled seeds that were germinated in the dark on a solid medium containing 75.0% of a hormone-free, modified B5 medium (Gamborg *et al.* 1968), 2.0% (m/v) sucrose and 0.80% (m/v) agar. Embryo formation started spontaneously via sub-cultured callus from callus-forming seedlings. The liquid cultures were carried out at 25±2 ºC

under a 16-h photoperiod in basal B5 medium. During maintenance and the studies, the embryogenic cultures were sub-cultured every three weeks and synchronized every six weeks by sieving. The growth rate of the culture was measured by cultivating 100 ± 5 mg of stock culture per flask for 70 d. Samples were taken three times during the first week and then once a week. For cryopreservation about 100 embryos (size < 250 μm) were transferred to cryotubes (Nunc A/S, Roskilde, Denmark) and pre-chilled B5 medium containing 1 M glycerol was added. The tubes were kept at +4 ºC for 1 h, with periodic swirling, and the temperature then lowered gradually to -35 ºC. After that the tubes were inserted into liquid nitrogen. The optimum carbon source for the cell line was studied using sucrose, glucose, fructose, and xylose. The concentration ranges used in the experiments were 0.50 - 4.5% (m/v) for sucrose, 1.1 - 6.3% (m/v) for glucose and fructose, and 0.88 - 4.3% (m/v) for xylose. The cultures were sub-cultured three times every three weeks. The effect of JA was studied by adding JA-containing medium to the culture flasks after 26 d of cultivation. The concentration range was from 0.1 to 500 μM and elicitation time 24 - 120 h.

For propagation, the embryogenic cell line was cultivated in an air-sparged, 5-dm^3 bioreactor (see **Figure 4**) with forced aeration (3 dm^{-3} min^{-1}) for 28 days. The reactor was filled with medium and inoculated with 10.0 g dm^{-3} of 3-week-old stock culture. Biomass production improvement was studied using a fed-batch procedure by adding 100 cm^3 of sterilized 60% (m/v) sucrose solution twice a week to the reactor. In the exponential growth phase of the culture, the plantlets were transplanted directly into a mixture of soil and vermiculite (30:70). The plantlets were kept under glass at + 18 °C for the first three weeks and then transferred to soil and greenhouse conditions. The plant material was separated from the medium by vacuum filtration, weighed and lyophilized for dry mass determination. The sucrose, glucose, and fructose concentrations of the media were determined using an enzyme-coupled colorimetric assay described by Hendrix (1993).

For the CELLOP studies **(IV)** the same culturing conditions were used as in **(III)**. Embryogenic cell lines of *A. archangelica* L. subsp. *archangelica* grown on a 75% (v/v) B5 basal medium, and *P. palustre* (L.) Moench (Vuorela *et al.* 1993) grown on 100% B5 basal medium, were used as reference. The experiments were carried out simultaneously with both cultures and 20 different experimental culturing media designed using the PRISMA culturing medium mixture design with eight replicates in each medium. Three nutrients, calcium (x_1), inorganic nitrogen (NO_3^-/NH_4^+) (x_2), and sucrose (x_3), were the explanatory variables in the design.

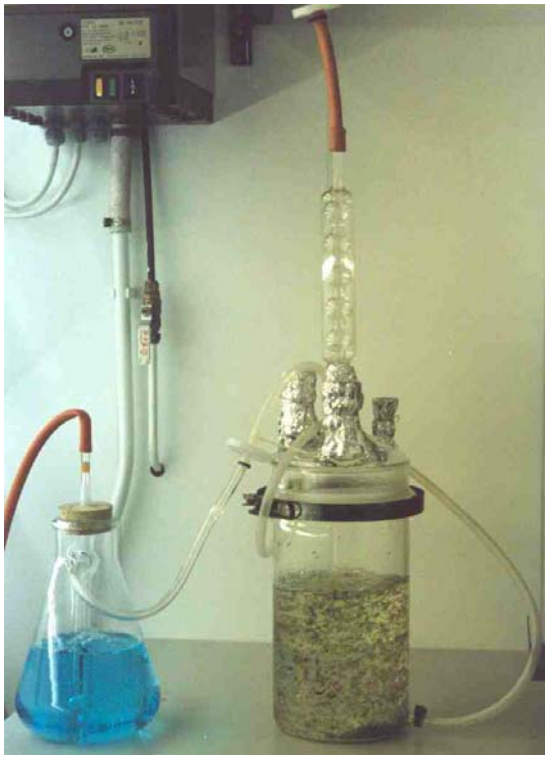


Figure 4. Air-sparged, 5-dm^3 bioreactor (Laborexin Oy, Helsinki, Finland; working volume 3 dm^3, height 300 mm, ø 230 mm) used in the propagation experiment (unpublished data).

8.5 Optimisation design for plant cell cultures (IV)

8.5.1 Mixture design and regression models

The CELLOP model can be visualised as a three-dimensional geometric design, a prism, in which the data points (P_d) in the horizontal part of the design represent different culturing medium mixtures. The data points can be depicted as three-number co-ordinates that represent the concentration ratios of the selected explanatory variables calcium, inorganic nitrogen (*i.e.* NO_3^-/NH_4^+), and sucrose, calculated so that the middle point 0.333:0.333:0.333 is the hormone-free Gamborg B5 basal medium. The vertical dimension symbolises the total strength of the medium mixtures. For the *A. archangelica* cultures the culturing media were diluted to 75% of the original strength.

Statistical models were constructed to predict the behaviour of the plant material in the experimental set-up using data from the growing experiments to find optimal conditions for growth and coumarin production. As the sum of the mixture component levels always equals one, the models for the responses (R_p) can be written in terms of two of the nutrients only. The regression models for the responses, linear, full quadratic or canonical model in

which the data points are described explicitly, were used to define the optimum in CELLOP. The best fitting regression model can be selected for each response variable separately.

8.5.2 Desirability functions

The desirability functions (Deming 1991) provide a means for defining the "desirable" and "undesirable" values of separate responses in the investigated system, and for combining them in an overall desirability function. The desirabilities for each response variable are defined separately, with a value of 0 for an unsatisfactory level of response and 1 when the desired level has been reached. The desirability functions of the individual response variables convert the calculated response values of dry mass (DM) (y_1) [g dm^{-3} (medium)], and coumarin concentration (y_2) [μg g^{-1} DM] into the unitless desirability values (D): $0 \leq D(R_p) \leq 1$. The desirability for the response, $D(R_p)$, is expressed with a logistic function $D(R_p)=1/1+e^{(-Rp-Rp0)/\delta}$, where R_p0 is the selected "mean response value" and δ is the "deviation" selected on the basis of the expected characteristics of the system. The overall desirability (D_o) is defined as the product, geometric mean $D_o = (d_1\ d_2....d_m)^{(1/m)}$ where $d_1\ d_2....d_m$ are the desirability functions of each response variable.

8.6 Statistical analysis

In **(II)** factor and cluster analyses were performed on the coumarin compositions in plants with SYSTAT 10.2 (SPSS; USA) software. The following expressions are used for P values of the correlation analysis results: significant $P < 0.05$, very significant $P > 0.01$, and highly significant $P > 0.001$. The effective temperature sums of the sampling locations used in the statistical analyses are based on the publication by Laaksonen (1979). The effect of JA on the coumarin composition in plant cell cultures **(III)** was studied statistically with SYSTAT 6.01 for Windows (SYSTAT Inc.; USA). As in Outinen *at al.* (1998), the data for CELLOP **(IV)** were processed by MATLAB version 5, student edition (Mathworks, Sherbon, MA, USA) with the data analysis toolbox on MATLAB (Profmath, Helsinki, Finland). A windowed graphical user interface was created for data handling and graphical assessment of the response surfaces.

9 RESULTS AND DISCUSSION

9.1 HPLC method development

Method development was initiated by carrying out seven isocratic runs of a *P. palustre* root extract with the eluent compositions suggested by the Turbo Method Development® programme. Due to the large number of peaks, automatic processing of the results with the Turbo Method Development® programme was insufficient. Thus the best resolution had to be selected manually. The selected isocratic eluent contained 60% of MeOH and 40% of 1% formic acid solution. Of the 1% formic acid and 10 mM ammonium acetate solutions, the acidic eluent proved to be superior with regard to peak shape and ionisation sensitivity in the initial LC/MS runs.

Two linear gradient runs with 20 - 95% MeOH in 20 and 60 min (see **Figures 5A** and **5B**) were performed. In total 36 peaks, separated and identified on the basis of the peak areas, was studied with an algorithm in DryLab® programme. As the a linear gradient of 29% - 100% MeOH in 15.3 min, proposed by DryLab®, did not lead to a satisfactory separation, it was manually segmented first to 0-12 min: 0-60% MeOH (linear gradient), 12-14min: 60% MeOH (isocratic), 14-25 min: 60-100% MeOH (linear gradient), and 25-35 min: 100% MeOH (isocratic), and then developed further to 0-6 min: 0-30% MeOH (linear gradient), 6-12 min: 30-60% (linear gradient); 12-14 min 60% MeOH (isocratic), 14-25 min: 60-100% MeOH; and 25-35 min: 100% MeOH (isocratic) (**Figure 5C**), enabling a satisfactory separation in 30 min. As the switch for formic acid from 1.0% to 0.1% did not markedly affect the retention time, peak shape or area, the plant extracts were analysed using the 0.1% formic acid solution as the aqueous phase.

The gradient system optimised with DryLab® was transferred to the HPLC system coupled to the MS apparatus operating in positive mode. The gradient was delayed by 2 min due to the decrease in dwell volume. Pure substances were analysed in order to obtain the retention time, UV spectrum, and MS spectrum data. Columbianadin, apterin, pimpinellin, psoralen, xanthotoxin, bergapten, isopimpinellin, imperatorin, isoimperatorin, oxypeucedanin, phellopterin ostruthol, osthol, and umbelliferone were identified by comparing the retention times and MS data of the sample runs with the respective data of pure substances. Columbianadin oxide, peulustrin/isopeulustrin, xanthotoxol, oxypeucedanin hydrate, isobyakangelicin angelate, osthenol, and umbelliprenin were identified on the basis of UV spectra, MS spectrum data, and the literature (for structures see **Figures 1-3**).

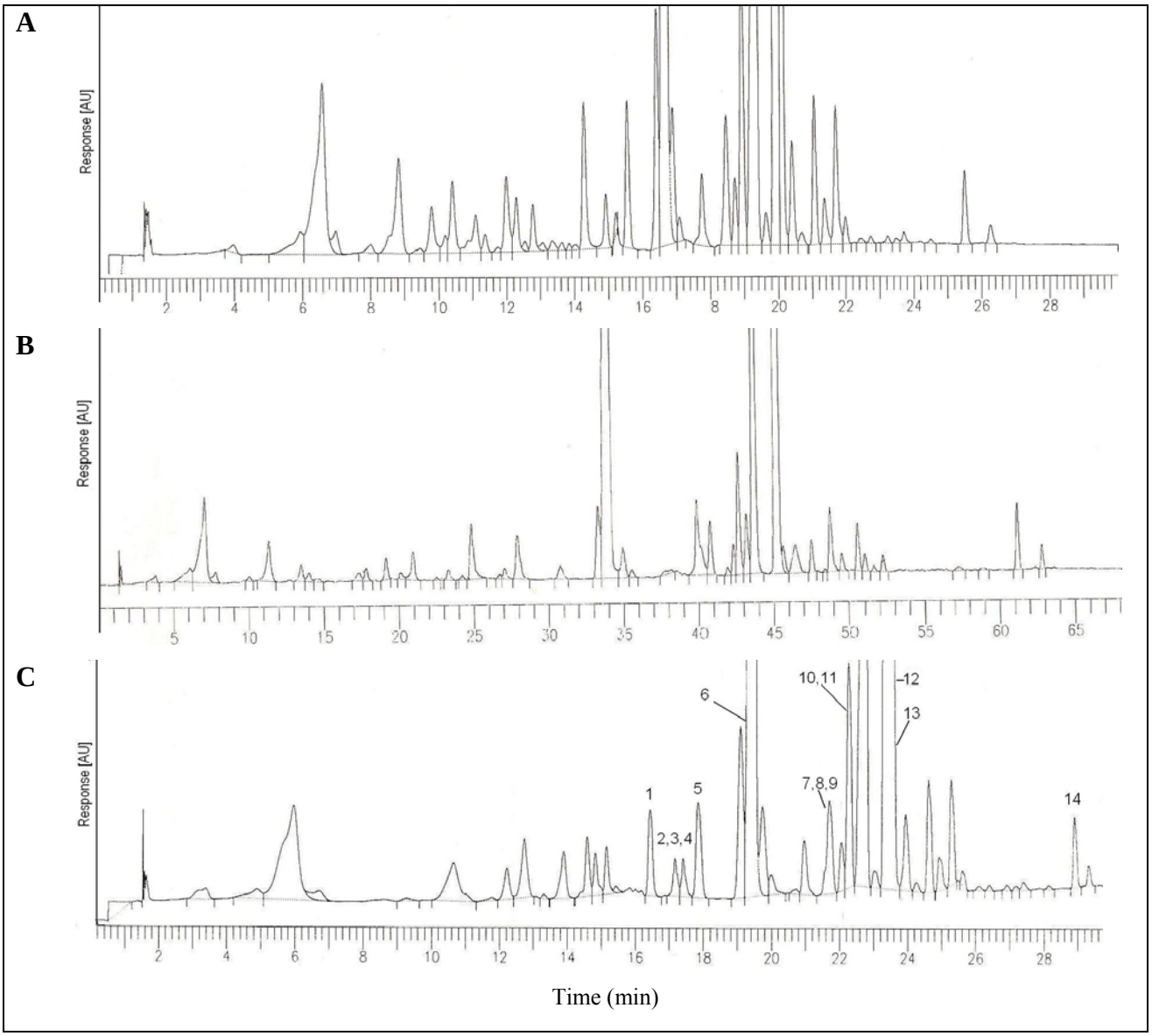


Figure 5.(A) Linear gradient run 20 min: 20-95% MeOH of the *P. palustre* root extract, **(B)** linear gradient run 60 min: 20-95% MeOH of the *P. palustre* root extract, and **(C)** segmented gradient run 0-6 min: 0-30% MeOH (linear gradient), 6-12 min: 30-60% (linear gradient); 12-14 min 60% MeOH (isocratic), 14-25 min: 60-100% MeOH; and 25-35 min: 100% MeOH (isocratic) of the *P. palustre* root extract (unpublished data). Identified coumarins were (1) oxypeucedanin hydrate, (2) xanthotoxin, (3) isopimpinellin, (4) pimpinellin, (5) bergapten, (6) oxypeucedanin, (7) peulustrin, (8) isopeulustrin, (9) isobyakangelicin angelate, (10) ostruthol, (11) columbianadin oxide, (12) isoimperatorin, (13) columbianadin, and (14) umbelliprenin.

9.1.1 *P. palustre*

A total of 13 coumarins were identified in the roots and 12 in the umbels of the *P. palustre* (see **Table 2**), the main coumarins being oxypeucedanin, ostruthol, columbianadin, and isoimperatorin, accounting for over 75% of the total coumarin content. The majority of the other detected peaks were less than 1% of the total peak area. Two of the main peaks, isoimperatorin and columbianadin, overlapped, but the extracted ion chromatogram (XIC) clearly showed two different compounds with almost the same retention time. Two peaks with the retention times of isopimpinellin and pimpinellin with matching *m/z* values at 247.4 ($[M+H]^+$) and 279.4 ($[M+H+MeOH]^+$) = were found in the *P. palustre* root samples (**Figure 6**). The XIC run revealed the presence of only one compound in the root with *m/z* values of 345.1 and 377.4, corresponding to $[M+H]^+$ and $[M+H+MeOH]^+$ of columbianadin oxide (MW = 344), respectively. So far, columbianadin oxide has only been reported in the fruits of *P. palustre* (Murray 1982). Only one peak with *m/z* values of 417.4 ($[M+H]^+$) and 445.3 ($[M+H+MeOH]^+$) was present, indicating the presence of isobyakangelicin angelate (MW = 416).

Of the previously reported compounds (Murray 1982), umbelliprenin was also found for the first time in the roots, as evidenced by the *m/z* values 367.5 ($[M+H]^+$) and 399.3 ($[M+H+MeOH]^+$). Umbelliprenin could not be detected with the HPLC method utilised in **(II)**. This study demonstrates the superiority of the gradient method over the isocratic method. The XIC run revealed traces of peulustrin/isopeulustrin with a *m/z* value of 445.3 ($[M+H]^+$) in the *P. palustre* roots. Further analysis would have required reference substances. The main compounds in the *P. palustre* root samples, oxypeucedanin and columbianadin, as well as umbelliprenin in the umbel samples, gave even double MeOH adducts $[M+H+2MeOH]^+$, the corresponding *m/z* values for oxypeucedanin being 351.4, for columbianadin 393.4, and for umbelliprenin 431.7. An XIC run of a *P. palustre* root sample between 216.8 and 217.8 amus revealed the existence of two compounds (**Figure 7A**). These gave *m/z* values of 217.5 and 249.3, which match the $[M+H]^+$ and $[M+H+MeOH]^+$ of both xanthotoxin and bergapten (**Figures 7B, C**). This finding was confirmed by matching retention times with the xanthotoxin and bergapten standards. Xanthotoxin has not been reported in *P. palustre* earlier (unpublished data).

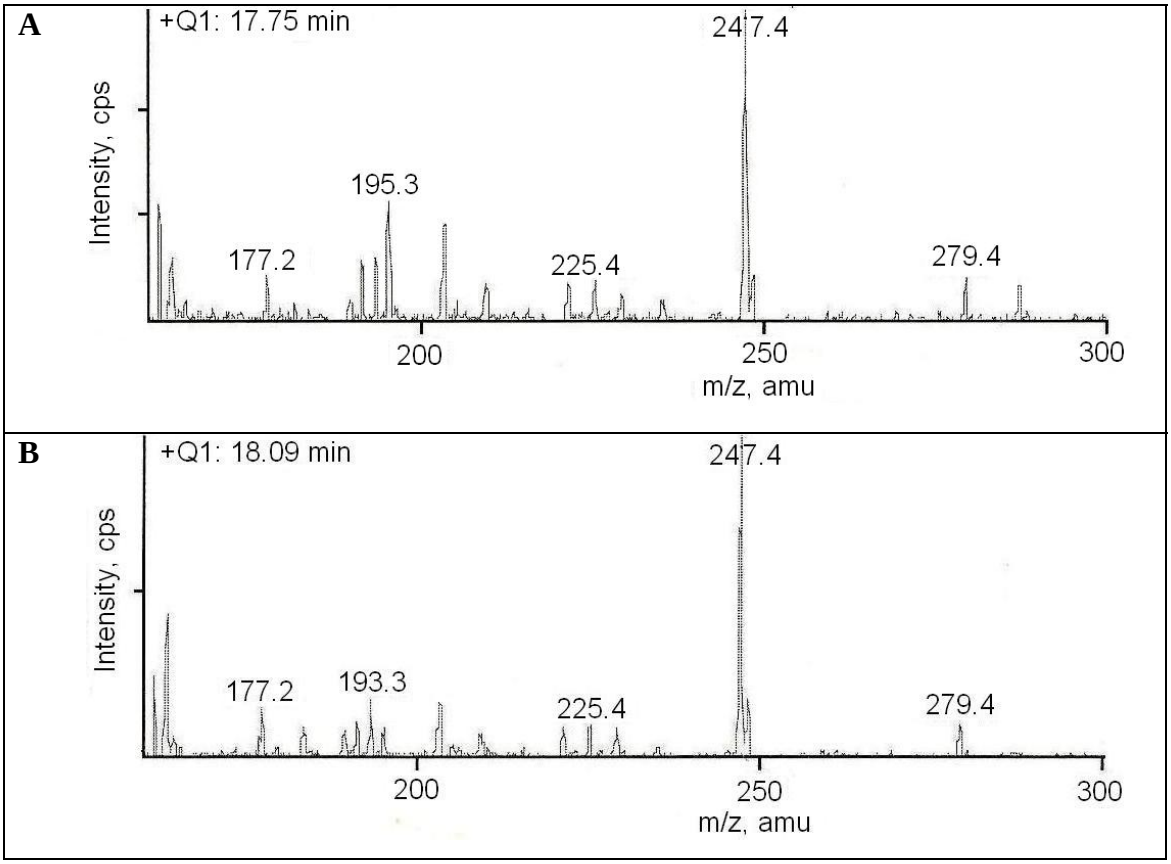


Figure 6. Mass spectrum of isopimpinellin (A) and pimpinellin (B) in the root samples of *P. palustre* (unpublished data).

Umbels of *P. palustre* have not been earlier analysed for coumarins. The main constituents in the umbels were peulustrin/isopeulustrin, ostruthol, columbianadin, isoimperatorin, and umbelliprenin. As in the roots, two peaks were found in the *P. palustre* umbels with retention times and *m/z* values of 247.4 ($[M+H]^+$) and 279.4 ($[M+H+MeOH]^+$) corresponding to those of isopimpinellin and pimpinellin. However, Hadaček *et al.* (1994) reported that isopimpinellin is present in the roots of other *Peucedanum* species.

The XIC (*m/z* = 445) of the umbel sample gave two overlapping peaks, indicating the presence of peulustrin and isopeulustrin. Due to the small difference in the molecular structure, co-elution of these coumarins is probable. Peulustrin has been reported in the roots, leaves, stems, and fruits of *P. palustre* (Hadaček 1989) and isopeulustrin in the fruits (Murray *et al.* 1982). Apterin tends to lose the sugar moiety (MW = 162) because the mass peak of the aglucone $[M-Glu+H]^+$ (*m/z* = 263) was present.

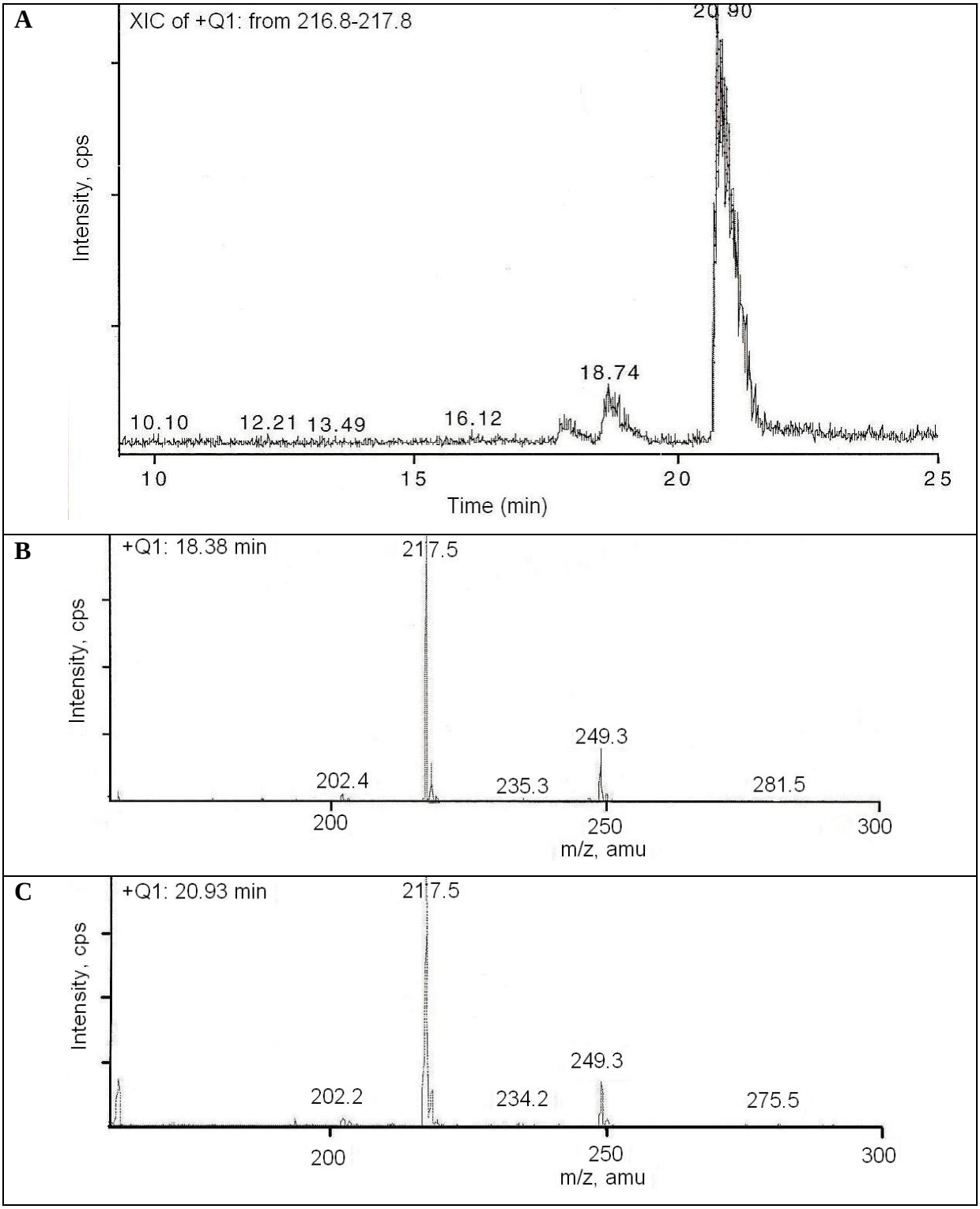


Figure 7. (A) XIC run of the root sample of *P. palustre* showing the presence of two compounds with a *m/z* value of 217.5 *i.e.* xanthotoxin and bergapten, (B) mass spectrum of xanthotoxin (*m/z* 217.5 = $[M+H]^+$, *m/z* 249.3 = $[M+H+MeOH]^+$), and (C) mass spectrum of bergapten (*m/z* 217.5 = $[M+H]^+$, *m/z* 249.3 = $[M+H+MeOH]^+$, unpublished data).

The XIC run from *m/z* 304.8-305.8 (oxypeucedanin hydrate MW = 304) gave three separate peaks, and the XIC run from *m/z* 286.8-287.8 (oxypeucedanin MW = 286) two peaks. These findings indicate the presence of isooxypeucedanin. This can also be deduced on the basis of previous reports (Murray *et al.* 1982).

The detection of pimpinellin and isopimpinellin in *P. palustre* is mainly due to the sensitivity of the MS instrument here applied for the first time in the analysis of *P. palustre*. The MS spectra of the main compounds in the *P. palustre* root samples, oxypeucedanin and columbianadin, as well as umbelliprenin in the umbel samples, were further characterised with *m/z* values corresponding to $[M+H+2MeOH]^+$ (*m/z* 351.4 for oxypeucedanin, 393.4 for columbianadin, and 431.7 for umbelliprenin).

9.1.2 *A. archangelica*

14 coumarins were identified in the roots, oxypeucedanin hydrate and oxypeucedanin being the main compounds (see **Table 1**). The XIC data also indicated the presence of osthenol, because a single peak was found that corresponded with the protonated molecule ($[M+H]^+$) of osthenol at *m/z* 231.3. Detectable amounts of angular furanocoumarins were not present in the analysed samples. Five coumarins were identified in the regenerated angelica leaves, the main compounds being xanthotoxin and isopimpinellin. Angular furanocoumarins were not detected. The coumarin concentrations in the regenerated leaves of *A. archangelica* were minute, but the concentration range of the identified compounds was also much smaller. This is most probably due to the fact that leaf size and seasonal variation have been found to affect the coumarin concentration of Apiaceae plants (Zobel and Brown 1990B, Trumble *et al.* 1992). Apart from the non-polar umbelliprenin, no simple coumarins were found in the regenerated leaves. Two peaks, with the same mass as oroselone (MW = 258), were found in both the roots and regenerated leaves of *A. archangelica*.

9.2 Coumarin composition of Finnish *P. palustre*

The analytical method allowed the identification of seven coumarins in *P. palustre, i.e.* oxypeucedanin hydrate, ostruthol, oxypeucedanin, bergapten, (iso-)peulustrin, isobyakangelicin angelate, and columbianadin. Distinguishing between structurally closely related peulustrin and isopeulustrin was not possible with the analytical method used in this study, and the compound is therefore referred to as (iso-)peulustrin in the text. Additionally,

six other compounds were also identified as coumarins but further structure elucidation proved to be difficult.

The total coumarin content varied markedly in different plant parts, being the highest in the umbels. The leaves and roots contained comparable amounts of coumarins. The lowest concentration was in the stems, being approximately one half of that in the root samples (see **Figure 8**). The main coumarins in the roots were oxypeucedanin and columbianadin. The stems contained predominantly (iso-)peulustrin, compound B, and oxypeucedanin. In the leaves the main components were (iso-)peulustrin and compound B. (Iso-)peulustrin was also the main component of the umbels. The other major coumarin in umbels was oxypeucedanin (see **Figure 9**).

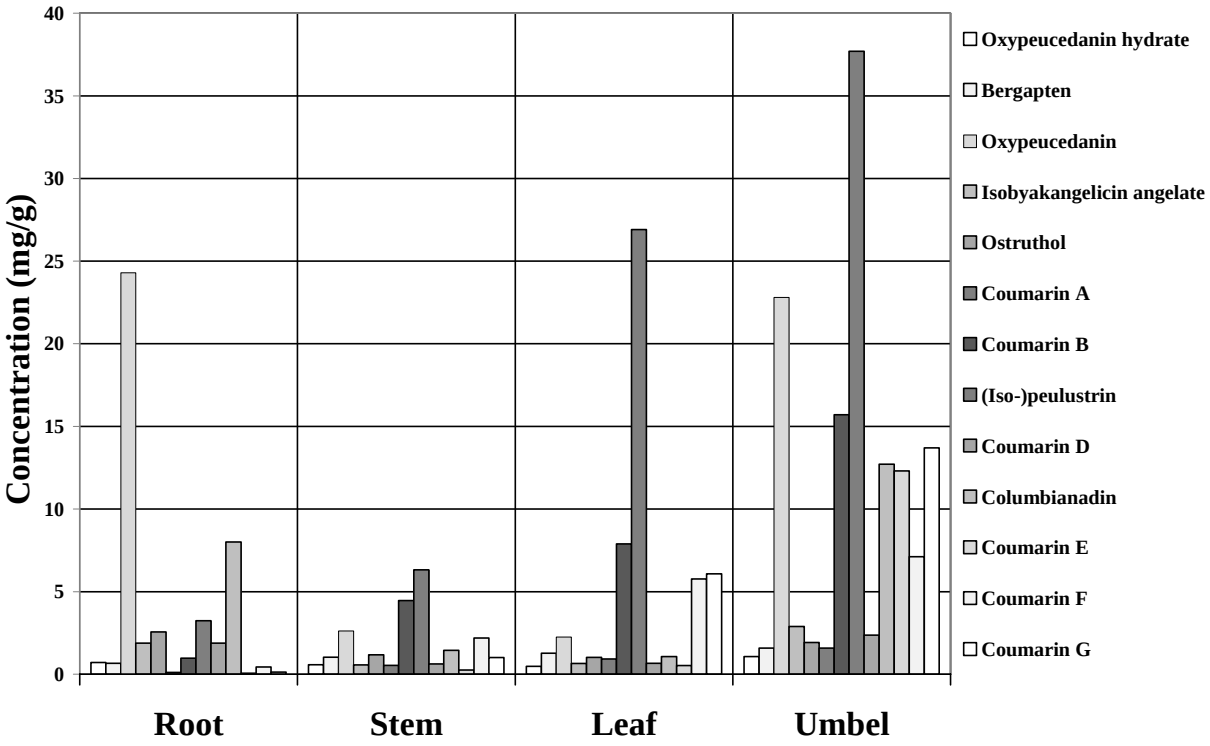


Figure 8. Mean concentrations (mg/g dry weight) of coumarins in the different plant organs of *P. palustre* (unpublished data).

The aerial parts mainly contained angular furanocoumarins, (iso-)peulustrin being clearly the largest component as it accounted for approximately 75% of the identified angular furanocoumarins. In the stem samples the amount of angular furanocoumarins was also higher, although they also contained approximately 20% of linear furanocoumarins (see **Figure 9**).

The highest total coumarin concentrations were found in southern Finland, and the concentrations decreased on moving towards the north. The negative correlations between the total coumarin concentration in umbels and leaves versus latitude were highly significant and that of the stems significant. Of all the coumarins, (iso-)peulustrin had the strongest negative correlation between concentration and latitude. The correlation was highly significant for umbels, leaves and roots, and for stems it was very significant. The columbianadin concentration in the roots correlated highly significantly with latitude and the correlation was positive, *i.e.* the columbianadin concentration in the roots increased towards the north.

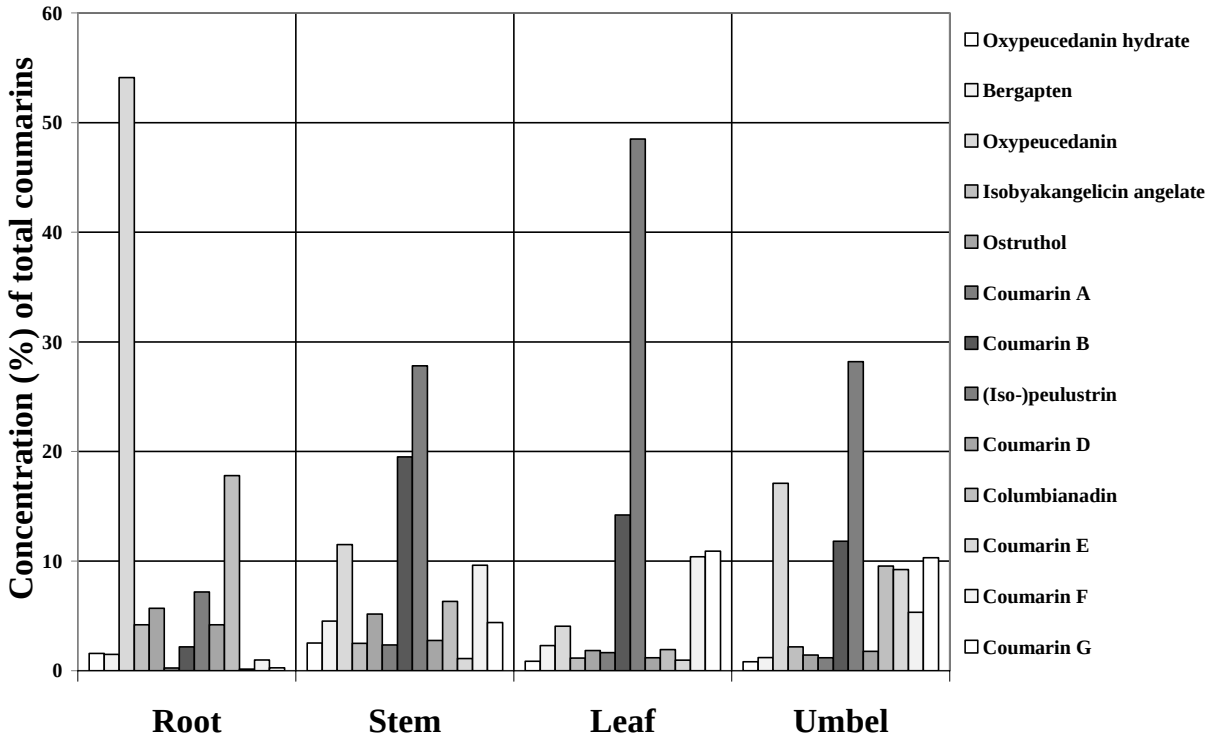


Figure 9. Proportion of coumarins out of the total coumarin content in different plant organs in *P. palustre* (unpublished data).

The effect of latitude of the growing location on the furanocoumarin content of *P. palustre* has not been studied earlier. The results indicate that the total coumarin concentration of *P. palustre* specimens growing in Finland decreases with latitude. The only plant organ without any significant correlation between the coumarin concentration and latitude was the root, and this may due to the storage function of subterraneous parts. In contrast to other detected coumarins, the concentration of the angular furanocoumarin columbianadin was found to increase with latitude. A similar qualitative, but not quantitative,

latitude-associated gradient in the concentration of foliar flavonoids and condensed tannins has been reported for white birch (*Betula pubescens* Ehrh.) growing in different locations around Finland (Stark *et al.* 2008). No correlation was found between the concentrations of total flavonoids and condensed tannins and latitude, even though the concentrations of quercetin derivatives correlated positively with latitude and the concentrations of apigenin and naringenin derivatives negatively with latitude, thereby cancelling each other out. For Scots pine (*Pinus sylvestris* L.), the terpenoid composition of a population growing in Finland was found to differ from populations growing in various parts of Turkey with regard to three monoterpenes, 3-carene, myrcene and terpinolene, but there were no significant differences in the concentrations of resin acids (Semiz *et al.* 2007). When studying the essential root oil of *A. arcangelica,* Ojala *et al.* (1986) also found that the concentration of 3-carene increased on moving from the south towards the north.

9.2.1 Correlations between plant organs

Coumarins in the roots had a positive, highly significantly correlation with a total of 12 coumarins present in the stems. The highest correlations were found between isobyakangelicin angelate in the roots and coumarin E in the stems, oxypeucedanin in the roots and stems, and oxypeucedanin hydrate in the roots and (iso-)peulustrin in the stems. Between the root and leaf coumarins, (iso-)peulustrin in the roots and coumarin G in the leaves had a highly significant positive correlation, and columbianadin in the roots and coumarin G in the stems a highly significant negative correlation. Root and umbel coumarins had the least number of highly and very significant correlations. Of all the aerial parts of *P. palustre,* the stem coumarins had clearly the highest correlations with the root coumarins, followed by leaf and umbel coumarins. Based on correlation matrix analysis, the coumarin content of the umbels and leaves were the most similar. Identification of the unknown coumarins detected in this study would be necessary in order to carry out a more comprehensive evaluation of the correlation, which would possibly provide a better insight into the biosynthesis of coumarins in *P. palustre*.

9.2.2 Correlations with the effective temperature sum

The total coumarin concentration correlated highly positively with the effective temperature sums of the respective sampling locations, the effective temperature sum ranging from 940 at the northernmost site at Iivaara, Kuusamo, to 1340 in Hamina, Virojoki, and

Rymättylä. The total coumarin concentrations of the umbels and leaves correlated positively to a highly significant degree with the effective temperature sum and the total coumarin concentration of the stems significantly. Based on correlation matrix analysis, the effective temperature sums clearly correlated with the coumarin concentrations of the aerial parts of *P. palustre,* but not with the roots. As earlier studies have shown, the coumarin content of *P. palustre* remains relatively constant during the first 40 days of the growing season (Kummala *et al.* 1993). The effect of effective temperature sum on the coumarin content cannot be explained by the plants being in different phoenological stages as all the collected plants were in the same growing phase and of similar size.

9.2.3 Cluster analysis

The existence of chemotypes could not be confirmed because cluster analysis did not reveal any division of the plant material into separate groups. This was mainly due to the high internal variation within the plant populations and to the variation between the plant populations. The main coumarins in the Finnish *P. palustre* populations, especially in the roots, corresponded well with the findings of Hadaček (1989). In both Finnish and Austrian populations the main coumarins in the roots were oxypeucedanin and columbianadin. The main difference was the higher concentration of (iso-)peulustrin in the Finnish plants and the higher concentration of ostruthol in the Austrian plants. The aerial parts, however, differed markedly from the results presented in Hadaček (1989). (Iso-)peulustrin was clearly the main coumarin in all the aerial parts of the Finnish plants, followed by oxypeucedanin and the unidentified coumarin B. The main coumarin in the stems and leaves in the Austrian plant populations, ostruthol, was found only in trace amounts in the Finnish plants. The results imply that, in general. Finnish populations differ from the Austrian population reported by Hadaček (1989).

The high concentration of the angular furanocoumarins, (iso-)peulustrin and columbianadin, is the most characteristic feature of Finnish *P. palustre* populations. One hypothesis explaining this phenomenon could be evolutionary response to endemic stress factors (Berenbaum and Zangerl 1998) caused by abiotic factors such as fungal and viral infections (*e.g.* Heath-Pagliuso *et al.* 1992, Ataga *et al.* 1993, Churngchow and Rattarasarn 2001, Repčák *et al.* 2001). It seems likely that several factors may contribute to the overall environmental stress experienced by the plants and the local furanocoumarin composition.

9.3 Somatic embryogenesis and propagation

The embryo formation capacity of the stock cultures of the *A. archangelica* cell line remained stable. The stock culture maintained its viability after cryopreservation. Transfer onto any new intermediate induction or development media was employed, which is often needed to achieve the subsequent developmental stages of embryogenesis or tissue formation (*e.g.* Laurain *et al.* 1996). No coumarins were detected in the isolated somatic embryo fractions (n = 4), but they were present in the developed plantlets. JA had no statistically significant effect on coumarin formation. JA and MeJa and other jasmonates are known to trigger coumarin production in intact Apiaceae plants, as well as in some plant cell cultures (Ellard-Ivey and Douglas 1996, Miksch and Boland 1996, Stanjek *et al.* 1997). Due to the low biosynthetic activity of the *A. archangelica* cell line the cells did not possess biosynthetic capacity to produce coumarins.

The *A. archangelica* culture grew slowly in the suspension culture, the highest dry mass (DM) and coumarin content being reached on day 56. The DM/FM ratio remained constant at 1/10 throughout the experiment. In corresponding studies, *P. palustre* cultures reached the maximum DM in four weeks (Vuorela *et al.* 1993) and in *A. sinensis* cultures already within 10 to 12 days (Tsay and Huang 1998). After eight weeks all the sugars had been utilized and the medium and plant material turned brownish and microscopic examination showed that cell had taken place.

9.3.1 Selection of carbon source

Sucrose, glucose, and xylose were tested to find the best carbon source. The DM in the sucrose-containing media varied between 132 ± 13 mg flask^{-1} to 371 ± 21 mg flask^{-1} (mean ± SD), reaching a maximum at 3.0% sucrose. The coumarin production of the culture was the highest, 5.72 ± 0.15 mg g^{-1} (mean ± SD), in the same medium. In *in vitro* studies on other plants in the Apiaceae family, callus cultures of *Pastinaca sativa* L. and *Ammi majus* L. were reported to produce up to 4.09 and 1.63 mg g^{-1} coumarins, respectively (Ekiert and Gomólka 2000, Ekiert 2000). It has earlier been shown that more developed stages of embryos of *P. palustre* produce larger amounts of coumarins (up to 5.62 mg g^{-1}), and are the highest (40.8 mg g^{-1}) and most diverse in the regenerated plant leaves (Härmälä *et al.* 1992A). Although growth was similar in the glucose media (from 156 ± 11 mg flask^{-1} to 296 ± 16 mg flask^{-1}) as in the sucrose media, coumarin formation was clearly lower (coumarin content 1.59 ±0.29 mg g^{-1} at 3.0% glucose). When the initial sucrose concentration was increased, the

appearance of the cultures gradually changed from greenish plantlets to white embryogenic cell clusters. On the other hand, when the media contained only traces of monosaccharides after cultivation, the *A. archangelica* embryos regenerated readily into plantlets. Glucose, fructose, galactose, mannose, maltose, raffinose, and stachyose have been shown to be suitable carbon sources for carrot cultures (Verma and Dougall 1977, Uozumi *et al.* 1991, Huang *et al.* 1993). In this case, fructose inhibited embryo formation and the cultures remained viable only at the lowest concentration (1.1%). This is also supported by the fact that, in the sucrose experiment, the residual amount of fructose was markedly high in the media with initial sucrose concentrations above 2% (m/v), indicating that the ability of *A. archangelica* cultures to utilise fructose is very limited (**Figure 10**). The embryos did not survive in xylose-containing media.

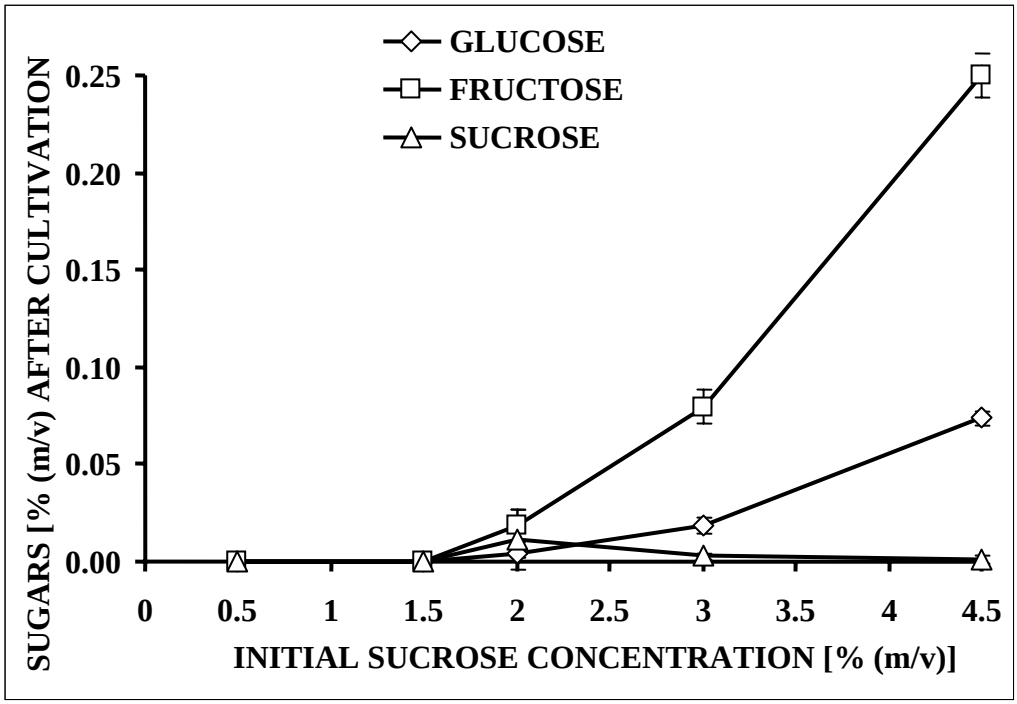


Figure 10. Accumulation of glucose, fructose and, sucrose in the growing media of *A. archangelica* embryogenic culture in relation to the initial sucrose concentration (unpublished data).

9.3.2 Propagation studies

In the bioreactor batch cultivation, the biomass production of the embryogenic *A. archangelica* culture was 2.3 g (FM) d^{-1}. As in the carbon source experiments, a higher sucrose concentration in the sucrose feeding experiments led to activation of embryogenesis

and the prevention of plantlet formation. Propagation studies were therefore carried out with plantlets from the bioreactor experiments without sucrose feeding. In this case plantlets were formed readily without any change of medium or addition of growth regulators. Shoots and roots developed simultaneously, and new embryo formation also continued at the same time. Similar behaviour has been reported in earlier work on *P. palustre* (Vuorela *et al.* 1993). After direct transfer from the bioreactor to unsterile conditions, 63% (n = 32) of the plantlets survived. The regenerated plants showed no morphological differences from normal plantlets as regards leaf, stem, or root formation (**Figure 11**). The coumarin content of the leaves of the regenerated 3-month-old plants (n = 9) varied between 1.95 and 8.06 mg g^{-1}, being somewhat lower than in intact plants from the same area as the mother plant, but the qualitative coumarin composition corresponded to that of intact plants (Ojala 2001). Seasonal changes in the coumarin content of Apiaceae plants are also known to be marked (Zobel and Brown 1990B, Trumble *et al.* 1992, Lois and Hahlbrook 1992). The stability of the *A. archangelica* cell line, the good viability of the *A. archangelica* plantlets in soil propagated in a simple air-sparged bioreactor, and the simplicity of direct transfer into pots, make the developed method a promising means for the economical production of large amounts of *A. archangelica* plants for field cultivation.

Figure. **11**. Propagated plantlets of *A. archangelica* immediately after transfer and after 3 weeks of cultivation (unpublished data).

9.4 CELLOP and optimisation of the growing conditions

On the basis of the average biomass (DM) and coumarin content, the "mean response value" Rp0 was set to 9.55 and "deviation" $\delta = 2.80$, $R_p0 = 9.55$; ($6.75 \leq R_p \leq 12.4$) and for coumarin content Rp0 = 5160; ($2890 \leq R_p \leq 7430$). The respective values for *P. palustre* were DM Rp0 = 10.75; ($5.85 \leq R_p \leq 15.65$) and the coumarin content Rp0 = 14790; ($8330 \leq R_p \leq 21250$). The full quadratic model was used for calculating the final results because it predicted the behaviour of the DM and coumarin content in both cultures better than the linear model (see **Tables 4** and **5**). The biomass production was easier to predict than the coumarin content, the r^2 values being clearly higher for the DM despite the regression model used.

The model suggested one clear overall optimum area for both cultures in the experiments. With *A. archangelica,* the overall optimum was located near the sucrose corner, in which the highest dry mass and coumarin content were reached, the dry mass being 24.6% and the coumarin content 40.5% higher than in the standard Gamborg B5 medium. The calculated overall optimum point with regard to the calcium, inorganic nitrogen, and sucrose concentrations of the medium was Ca^{2+} 0.47 mM, NO_3^- 5.06 mM, NH_4^+ 0.40 mM, and sucrose 96.25 mM, giving a total desirability of $D_o = 1.0$. This is in good agreement with the observations in **(III)**.

For *P. palustre,* the overall optimum area was between the calcium and sucrose corners. Of the separate responses in the model, the DM maximum was close to the sucrose corner, while the coumarin content was the highest between the calcium and sucrose corners. The DM was 61.8% higher in the optimised medium than in the standard Gamborg B5 medium. The highest coumarin content exceeded the reference by 58.1%. The amount of nitrogen needed for growth and coumarin production was markedly low. Thus the calculated optimum with regard to the three variables was 1.60 mM Ca^{2+}, 2.84 mM NO_3^-, 0.23 mM NH_4^+, and 85.10 mM sucrose, giving a total desirability of $D_o = 1.0$.

The variation in the concentrations of the explanatory variables clearly affected embryo formation and differentiation (shoot/root ratio). The 1.25 mM calcium concentration increased embryo formation of the *A. archangelica* cultures the most. Reports on other embryogenic cell lines such as carrot (Overvoorde and Grimes 1994) and *Hevea brasiliensis* (Muell Arg.) (Etienne *et al.* 1997) support this finding. In the *P. palustre* experiments, the inorganic nitrogen concentration was the key factor for embryo formation. The same has also been reported for *Betula bendula* Roth cultures (Nuutila *et al.* 1991). However, sugar played

an important role in embryo formation with both of the cultures, as noted earlier (Jeannin *et al.* 1995, **(III)**). CELLOP enables emphasizing the required or desirable features of the examined material such as morphology, *i.e.* the shoot/root ratio, embryogenic activity or biosynthetic activity. The explanatory variables in this study make it possible to use the experimental set-up, *e.g.* in the optimisation of *in vitro* propagation procedures or in the development of two-stage culturing methods for bioprocesses (**(III)**, Chen *et al.* 2003).

9.4.1 Development of the experimental design

The total duration of the optimisation experiment is critical for slowly growing cultures. Methods suitable for such cultures have been developed *e.g.* by Tammisola *et al.* (1993), but the number of experimental steps may still easily increase, *e.g.* the duration of a 5-step study with 3 sub-culturing phases is over 10 months. A differentiated culture, especially the *A. archangelica* line, is markedly affected by seasonal changes **(III)**. Thus a series of experiments lasting longer than 10 months is easily affected by this phenomenon, and a faster approach such as CELLOP is essential.

The reduction in the number of experimental data points from 20 to 13 and replicate flasks from 8 to 3 did not have any marked effect on the overall optimum or desirability (see **Tables 4** and **5**). However, a further reduction in the number of data points or replicate flasks proved to be impossible. The model provides a means for systematic optimisation of the growth medium for plant cell cultures within a relatively short period of time. The results from both **(III)** and **(IV)** lead to the concept of a two-phase cultivation system, in which cultivation is initiated with sucrose feeding in order to rapidly obtain a large amount of embryogenic material continued by cultivation in a low-concentration sucrose-medium that activates the regeneration of embryos into plantlets. Although the plants from which the cultures were derived are closely related, they still showed significant differences both in growth and coumarin production, as well as in embryo formation, thus making individual optimisation necessary for each culture. The developed model also enables the determination of absolute concentrations of the chosen explanatory variables for new cultures by using the vertical dimension of the prism.

Table 4. Effect of number of experimental data points, replicate flasks, and regression models, on r^2 of dry mass, on $r^2$of coumarin content, overall optima, and desirability values of *A. archangelica* cultures (unpublished data).

Data points	Model	Flasks	r^2 DM	r^2 coum.	Overall optimum	D_o
20	Linear	8	73.8	65.5	0,145; 0,002581; 0,8524	1
		5	75.2	67.32	0,145; 0,002581; 0,8524	1
		3	70.42	66.38	0,145; 0,002581; 0,8524	1
		2	73.45	61.69	0,145; 0,002581; 0,8524	1
		1	64.08	54.04	0,145; 0,002581; 0,8524	1
	Full quadratic	8	82.01	69.61	0,1857; 0,08099; 0,7333	1
		5	81.85	69.86	0,1857; 0,08099; 0,7333	1
		3	82.95	70.73	0,1857; 0,08099; 0,7333	1
		2	82.52	67.53	0,1623; 0,1827; 0,6549	1
		1	80.04	64.75	0,1677; 0,2676; 0,5647	1
13	Linear	8	75	72.18	0,145; 0,002581; 0,8524	1
		3	69.23	70.16	0,145; 0,002581; 0,8524	1
		2	73.15	65.52	0,145; 0,002581; 0,8524	1
		1	62.02	56.13	0,145; 0,002581; 0,8524	1
	Full quadratic	8	83.87	75.27	0,1857; 0,08099; 0,7333	1
		3	84.79	74.17	0,1857; 0,08099; 0,7333	1
		2	84.41	70.87	0,08099; 0,1857; 0,7333	1
		1	84.61	67.08	0,08099; 0,1857; 0,7333	1
7	Linear	8	73.92	68.97	0,145; 0,002581; 0,8524	1
		3	69.19	70.36	0,1857; 0,08099; 0,7333	1
		2	76.08	66.43	0,1857; 0,08099; 0,7333	1
		1	66.85	47.03	0,145; 0,002581; 0,8524	1
	Full quadratic	8	83.43	75.16	0,002581; 0,145; 0,8524	1
		3	87.91	85.9	0,362; 0,5172; 0,1208	1
		2	90.87	88.06	0,5027; 0,3982; 0,0946	1
		1	98.23	97.83	0,362; 0,5172; 0,1208	1

Table 5. Effect of number of experimental data points, replicate flasks, and regression models, on r^2 of dry mass, on $r^2$of coumarin content, overall optima, and desirability values of *P. palustre* cultures (unpublished data).

Data points	Model	Flasks	r^2 DM	r^2 coum.	Overall optimum	D_o
20	Linear	8	81.28	46.91	0.145; 0.002581; 0.8524	0.9996
		5	79.31	48.40	0.145; 0.002581; 0.8524	0.9993
		3	83.25	43.23	0.4819; 0.01025; 0.5078	0.9999
		2	79.00	45.36	0.145; 0.002581; 0.8524	0.9992
		1	79.3	40.63	0.499; 0.01198; 0.489	0.9999
	Full quadratic	8	90.37	56.37	0.4796; 0.03407; 0.4863	1
		5	89.03	60.41	0.4796; 0.03407; 0.4863	1
		3	90.37	56.30	0.4796; 0.03407; 0.4863	1
		2	87.89	61.46	0.4796; 0.03407; 0.4863	1
		1	87.90	62.85	0.5381; 0.01691; 0.445	1
13	Linear	8	79.62	49.76	0.145; 0.02581; 0.9524	0.9992
		3	83.71	43.78	0.5058; 0.0009417; 0.4932	0.9997
		2	79.45	47.53	0.5414; 0.0002574; 0.4583	0.9998
		1	78.95	46.34	0.5444; 0.000271; 0.4554	0.9997
	Full quadratic	8	93.02	66.75	0.4796; 0.03407; 0.4863	1
		3	94.21	66.92	0.6064; 0.01308; 0.3805	1
		2	92.98	72.44	0.4796; 0.03407; 0.4863	1
		1	92.21	80.32	0.4796; 0.03407; 0.4863	1
7	Linear	8	69.8	37.71	0.08315; 0.006804; 0.91	0.9999
		3	78.98	27.09	0.08315; 0.006804; 0.91	0.9994
		2	74.97	33.87	0.08315; 0.006804; 0.91	0.9997
		1	74.62	16.83	0.000701; 0.000226; 0.999	0.9737
	Full quadratic	8	93.86	58.83	0.1677; 0.2676; 0.5647	0.9999
		3	96.9	79.94	0.1725; 0.2665; 0.5609	1
		2	99.39	79.92	0.1725; 0.2665; 0.5609	1
		1	99.78	99.95	0.1603; 0.3718; 0.4679	0.9996

10 CONCLUSIONS

MS detection with automated optimisation of chromatographic conditions enables, compared to earlier techniques, the detailed analysis of complex plant samples with closely related compounds. The amount of sample material required is also relatively low. Fast and efficient methods provide a valuable tool for more efficient processes when screening plant material in the search for new medicines.

Several statistically significant correlations were found between different coumarins in the different plant organs. The Finnish *P. palustre* population did not show any signs of containing different chemotypes. This may be due to the high variability of the coumarin composition. However, it clearly differed from the Austrian population. Climatic factors, *e.g.* effective temperature sum, affected only the aerial parts of *P. palustre*.

Somatic embryogenesis proved to be a useful method for producing healthy plants of *A. archangelica*, which are known to be difficult to propagate due to the poor germination ability of the naturally produced seeds. The use of a simple bioreactor makes the method useful for the large-scale production of *A. archangelica* plants. Further studies are, however, needed to ensure the genetic stability of the culture.

The optimisation model, "CELLOP" utilising desirability functions, which has earlier been utilised in the optimisation of chromatographic conditions, enabled systematic optimisation of the cultivation conditions of plant cell cultures in a relatively short period of time compared to classical optimisation approaches. What should not be forgotten, however, is that not only the relative quantities of the optimised nutritional factors but also their absolute quantities should be taken into account.

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