Seasonal Variations in the Deoxypodophyllotoxin Content and Yield of *Anthriscus sylvestris* L. (Hoffm.) Grown in the Field and under Controlled Conditions

Oktavia Hendrawati,[†] Herman J. Woerdenbag,[‡] Jacques Hille,[§] Wim J. Quax,[†] and Oliver Kayser*[,†,⊥]

[†]Department of Pharmaceutical Biology and [‡]Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Groningen, The Netherlands

[§]Department of Molecular Biology of Plants, University of Groningen, Haren, The Netherlands

[⊥]Technical Biochemistry, Technical University Dortmund, Dortmund, Germany

ABSTRACT: Deoxypodophyllotoxin (DPT) is the main lignan in *Anthriscus sylvestris*. For this study two sets of experiments with 16 plants and seeds, collected from a wide geographical range, were carried out. The DPT content in roots was significantly lower ($p < 0.05$) when the plants were cultivated in a non-native environment. For field-grown plants the highest DPT content was found in March (second year): 0.15% w/w (dry weight) in roots; 0.03% w/w in aerial parts. For plants grown in the climate room, the highest concentration (0.14% w/w) was observed in April (second year) in the roots and in July (first year) in the aerial parts (0.05% w/w). For the isolation of DPT, roots are the most suitable part. The best harvest times are March (second year) for outdoor plants and April (second year) for indoor plants when height content and adequate biomass give the optimal DPT yield.

KEYWORDS: lignan, deoxypodophyllotoxin, podophyllotoxin, *Anthriscus sylvestris*, environmental factors, plant cultivation, biosynthesis, etoposide, teniposide

■ INTRODUCTION

Anthriscus sylvestris (L.) Hoffm. (Apiaceae) is a common wild plant in northwestern Europe, in parts of North America, Africa, Asia, and New Zealand.[1−3] The dried roots are used in Korean and Chinese traditional medicine for the treatment of various diseases, including bronchitis, and as an antipyretic, cough remedy, and analgesic herbal drug.[4−6] The plant accumulates considerable amounts of lignans, which are held responsible for the biological activity.[7]

Deoxypodophyllotoxin (DPT) (Figure 1) is considered to be the plant's main lignan constituent.[7] It has pharmacological properties such as antiproliferative, antitumor, antiviral, antiinflammatory, antiplatelet aggregation, and antiallergic related activities in disorders including asthma.[8−16] The number of studies of DPT mechanism of action against cancer cells[17] and other pharmacological properties is growing.[4,18−21] It also has insecticidal properties.[22,23] The closely related podophyllotoxin (Figure 1) is used for the semisynthesis of the cytostatic agents etoposide and teniposide. These drugs are used for the treatment of lung and testicular cancers, Ewing's sarcoma, lymphoma, glioblastoma, and nonlymphomatic leukemia.[24]

In several plant species, DPT serves as a biosynthetic precursor for podophyllotoxin.[25,26] Feeding experiments with cultures of undifferentiated plant cells (*Linum album*) or fungi (*Penicillium* F-0543 and *Aspergillus niger*) have shown that DPT can be converted into podophyllotoxin.[25,27]

To date, podophyllotoxin is obtained by extraction of the rhizome of *Podophyllum* species. The availability of *Podophyllum* species is limited,[28] and the increasing demands of podophyllotoxin jeopardize the natural sources. *Podophyllum* species are listed on the endangered species list in India.[29,30] Soon, the availability of podophyllotoxin will be a major bottleneck in supplying pharmaceutical needs.

Preliminary studies focusing on the question of which factors may influence the production of DPT in *A. sylvestris* suggested that environmental factors highly determine the lignan profile and content.[31,32] It has also been reported that DPT content differs at least 2-fold between low and high altitudes both in aerial (0.13% dw at 900 m, 0.33% dw at 1200 m) and in root parts (0.38% dw at 900 m and 0.78% dw at 1200 m) of *A. sylvestris*.[21] Different populations of *A. sylvestris* yielded different lignan patterns, and there was no clear genetic factor determining the lignan profiles when seeds from different locations were grown under identical conditions.[31,32] A major drawback of that study was the limited number of plants. Here we conducted a biannual experiment with a higher number of collected plants and seeds from a wider geographical range. The plants from the previous studies were included as well (Table 1). All plants were grown under identical field conditions and in a climate room. DPT was the main important compound and was therefore used as the biosynthetic marker. The field resembles natural weather conditions, whereas the climate room resembles controlled cultivation.

The first objective of this study is to determine the DPT content of native and non-native *A. sylvestris* grown in the field and in a climate room to get insight into the production rate of DPT in *A. sylvestris*. Second, we aimed to study the influence of growing conditions, both outdoor and indoor, on the DPT production. Furthermore, it is important to identify rate-limiting ecological factors for optimal growing conditions of *A. sylvestris* for DPT production.

■ MATERIALS AND METHODS

Chemicals. DPT was a gift from Dr. M. Angeles Castro (Salamanca University, Spain) and was synthesized chemically. The identity and purity of DPT were checked by using HPLC (100%). Acetonitrile and methanol were of HPLC grade from Biosolve (Valkenswaard, The Netherlands). Dichloromethane was from Fisher Scientific (Landsmeer, The Netherlands).

Plant Material. The fruits (ripe mericarps, hereafter called seeds) and plants of *A. sylvestris* L. were obtained from different sources (Table 1). Voucher specimens are deposited in our department coded Asylv2007-1−16. The identity of the seeds was verified, and samples were stored in the reference collection of the Groningen Institute of Archaeology. *A. sylvestris* plants were collected at the flowering stage between May and June 2007, from locations in The Netherlands and other European countries (Table 1), and used for analysis. From the same plants and origin, seeds were collected. Plants were grown from these seeds (identical conditions) in the Botanical Garden de Kruidhof (Buitenpost, The Netherlands, 53° 15′ 52″ N, 06° 07′ 41″ E) and sampled in 2009. The roots were separated immediately from the aerial parts. The plant material was dried at room temperature prior to grinding and extraction.
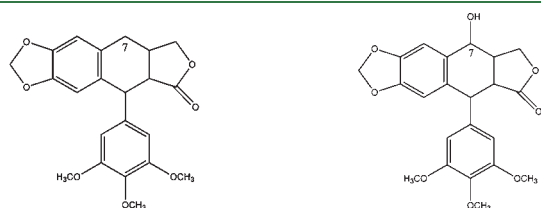
Plant Cultivation in the Field (Outdoor). The seeds were sown in December 2007 on soil in trays for cold stratification at the Botanical Garden de Kruidhof and germinated in February 2008. These seedlings were transferred to the field in May 2008. The field samples were collected in the second year (2009) on a monthly basis, from March until August. The data (Table 2) of the weather conditions in The Netherlands during this experiment in 2007−2009 were taken from

Deoxypodophyllotoxin   Podophyllotoxin

Figure 1. Chemical structures of deoxypodohyllotoxin and podophyllotoxin.

KNMI (The Netherlands Meteorology Institute) Website (www.knmi.nl).

Plant Cultivation in the Climate Room (Indoor). The seeds were sown in December 2007 on soil at the Botanical Garden de Kruidhof and germinated in February 2008. The seedlings were transferred into individual pots and placed under a day−night regimen (16 h light and 8 h dark) at 20−22 °C in May 2008. In the first year (2008), samples were collected in June, July, and August. During the subsequent winter period (September 2008−February 2009), the plants were placed in an open greenhouse to induce a cold dormancy period. In March of the second year (2009), they were placed back into the climate room, and samplings were continued every month, from March until August.

Sample Preparation and Extraction Method. All samplings were from three individual plants (triplicate) and randomly selected. The samples were divided into aerial part and root part. Subsequently, the biomass (fresh weight) of the aerial and root parts was weighed for every plant. The biomass data were an average of three individual plants. Samples from the field and the climate room experiments were freeze-dried and pulverized for analysis. The fresh weight/dry weight ratio was calculated on the basis of an average of six plants. The ratio for the aerial part was 0.1891 and for the root part, 0.2840. The yield was calculated based on DPT concentration and total dry weight of the biomass per plant. The extraction method was as described by Koulman et al.[31] Briefly, 100 mg of dried plant material was weighed in a sovirel tube. A 2.0 mL portion of 80% methanol was added, and the mixture was sonicated during 1 h. Subsequently, 4.0 mL of dichloromethane and 4.0 mL of H_2O were added. The mixture was vortexed and centrifuged at $1000g$ for 5 min. The aqueous layer was discarded, and 2.0 mL of organic layer was transferred into a 2 mL Eppendorf tube. The organic layer was left in the fume hood until dried. The residue was redissolved in 2.0 mL of methanol and filtered over a 0.45 μm HPLC syringe filter (nylon). The samples were submitted to HPLC analysis.

HPLC Analysis. The HPLC analysis was as described by Vasilev et al.[33] with some modifications. We used a Shimadzu-VP system consisting of a LC-10AT pump, a Kontron 360 autosampler, a SPD M10A DAD detector, a FCV-10AL low-pressure gradient mixer, a SCL-10A system controller, and a FIAtron system CH-30 column heater, operated with LC Solution software, version 1.2. The column used was a Zorbax Eclipse C18 (150 × 4.6 mm, 5 μm), together with a Phenomenex guard cartridge C18 (4 × 3 mm; Phenomenex, Bester, The Netherlands). The detection

Table 1. Origins of the Collected *Anthriscus* Plants and Seeds

no.	origin	country	location		amsl[a]	collector
1	Nieuwegein[b]	The Netherlands	52° 00′ 50″ N	05° 06′ 68″ E	1	Oktavia Hendrawati
2	Olst[b]	The Netherlands	52° 32′ 97″ N	06° 10′ 21″ E	3	Oktavia Hendrawati
3	Diever[b]	The Netherlands	52° 85′ 95″ N	06° 32′ 91″ E	9	Oktavia Hendrawati
4	Groningen[b]	The Netherlands	53° 11′ 34″ N	06° 37′ 04″ E	1	Oktavia Hendrawati
5	Buitenpost	The Netherlands	53° 15′ 52″ N	06° 07′ 41″ E	1	Jan W. Zwart
6	Oudeschans	The Netherlands	53° 08′ 19″ N	07° 08′ 34″ E	1	Rene J. T. Cappers
7	Bonn	Germany	50° 45′ 04″ N	07° 06′ 39″ E	60	Frank Klingenstein
8	Tartu	Estonia	58° 36′ 60″ N	26° 69′ 25″ E	79	Christina Birnbaum
9	Coventry	United Kingdom	52° 24′ 03″ N	01° 30′ 40″ W	76	Dalila Martins
10	Essex	United Kingdom	na	na	38	Kew Garden, accession no. 0039420
11	Suffolk	United Kingdom	na	na	na	Kew Garden, accession no. 0053121
12	Enebyberg	Sweden	59° 26′ 06″ N	18° 01′ 48″ E	20	Renata Alnsatter
13	Reykjavik	Iceland	66° 21′ 25″ N	21° 71′ 29″ W	15	Sigurdur H. Magnusson
14	Egillstafr	Iceland	64° 59′ 57″ N	14°17′ 22″ W	34	Sigvardur Eirnarsson
15	Hrisey	Iceland	66° 01′ 03″ N	18° 23′ 69″ W	5	Bjarni E. Gudleifsson
16	Mogilsa	Iceland	66° 21′ 25″ N	21° 71′ 29″ W	65	Sigurdur H. Magnusson

[a] Above mean sea level (m). [b] Same locations as in the preliminary studies.[31,32]

Table 2. Data on Rainfall, Temperature and Duration of Sun in the Netherlands in 2008 and 2009 (www.knmi.nl)

	month											
	1	2	3	4	5	6	7	8	9	10	11	12
rainfall 2007 (mm)	104	68	85	0	138	90	161	42	97	32	58	77
rainfall 2008 (mm)	96	39	92	34	33	40	127	114	100	92	91	24
rainfall 2009 (mm)	54	55	48	20	65	54	107	47	34	90	120	84
temperature 2007 (°C)	7.1	6.0	8.0	13.1	14.1	17.5	17.0	17.1	13.8	10.1	6.9	3.8
temperature 2008 (°C)	6.5	5.1	5.9	8.9	15.7	16.5	18.1	17.4	13.6	10.1	6.9	2.4
temperature 2009 (°C)	0.8	3.3	6.3	12.2	13.9	15.6	18.1	18.5	15	10.7	9.5	2.2
duration of sun 2007 (h)	50	57	157	284	198	172	193	199	116	130	62	72
duration of sun 2008 (h)	50	128	117	185	260	233	182	163	147	134	54	85
duration of sun 2009 (h)	101	48	142	227	236	232	233	226	160	123	51	60
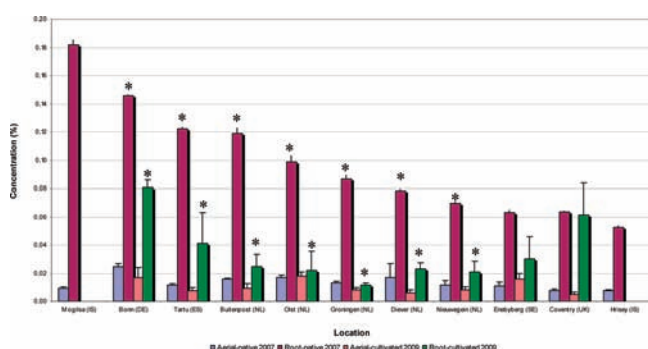
Figure 2. Deoxypodohyllotoxin content of flowering *A. sylvestris* collected in their native location and flowering *A. sylvetris* cultivated from their native fruits in the same field (Botanical Garden de Kruidhof, The Netherlands). Seeds from Hrisey and Mogilsa did not germinate; therefore, no data could be recorded in 2009.

wavelength was 240 nm. Mobile phases A [H_2O/ACN (95:5)] and B [ACN/H_2O (95:5)] were both in 0.1% formic acid and 2 mM ammonium formate. The injection volume was 5 μL. The flow rate was 1 mL/min using a gradient program of 30 min consisting of 1 min of B 30%, followed by a linear gradient until 15 min, B 90%; at 20 min, B 90%; at 25 min, B 30%; and at 30 min, B 30%. Concentrations of metabolites were calculated using a calibration curve established with the reference compound (DPT).

Statistical Analysis. Statistical calculations were carried out with SPSS 16.0. Pearson correlation was used for correlation analysis, and *t* test was used for significant testing. *p* values of <0.05 were considered to be significant.

■ RESULTS

DPT Content of Native and Cultivated *Anthriscus* Plants. The DPT content of *Anthriscus* plants from their native location was compared to that of the plants from the seeds of the native location, grown in the Botanical Garden de Kruidhof. The native *A. sylvestris* from Iceland (Mogilsa) contained the highest DPT production in the root part (0.182%), and the native *A. sylvestris* from Germany (Bonn) contained the highest DPT production in the aerial part (0.025%). For the cultivated plants, the highest DPT production in the root part was the *A, sylvestris* from Bonn (0.081%) and Coventry (0.062%), whereas that in the aerial part was the *A. sylvestris* from Olst (0.018%). The results are shown in Figure 2.

The DPT content of all field plants cultivated in The Netherlands in 2009 was used for the correlation analysis. There was a significant negative correlation between the DPT content in the aerial part of the field plants with the rainfall ($r = -0.624, p < 0.01$), with the duration of sun ($r = -0.402, p < 0.01$), and with the temperature ($r = -0.643, p < 0.01$). There was also a significant negative correlation between the DPT content in the root part of the field plants with the duration of sun ($r = -0.611, p < 0.01$) and with the temperature ($r = -0.599, p < 0.01$). In the climate room plants, there was a significant negative correlation between the DPT content in aerial part with the rainfall ($r = -0.430, p < 0.01$) and with the temperature ($r = -0.190, p < 0.01$). There was also a significant negative correlation between the DPT content in the root part with the duration of the sun ($r = -0.228, p < 0.01$), with the temperature ($r = -0.464, p < 0.01$), and with the rainfall ($r = -0.362, p < 0.01$). There was a positive correlation between the DPT contents in the aerial and in the root of the field plants ($r = 0.315, p < 0.01$) and the climate room plants ($r = 0.374, p < 0.01$). There was a significant positive correlation between the total yield of DPT in the aerial part of the field plants with the duration of sun ($r = 0.221, p < 0.01$). There was a significant negative correlation between the total yield of the DPT in the root part of the field plants with the duration of sun ($r = -0.573, p < 0.01$) and temperature ($r = -0.507, p < 0.01$). Thus, a lower temperature and less sun result in a higher DPT production in *A. sylvestris*.

DPT Content of the Field Plants (Outdoor Plants). The experiments in the field with *A. sylvestris* plants grown from seeds of the various origins (uncontrolled condition–outdoor plants) and in the climate room (controlled condition–indoor plants) were carried out in parallel. The DPT content variation over the developmental stages was measured and compared both for the aerial (Figure 3A) and the root parts (Figure 3B). *A. sylvestris* is a biannual plant; therefore, we started sampling in the second year when the plants develop biomass and flower. The DPT content in the roots of the field plants was up to 0.15% and in the aerial parts, up to 0.03%. The highest DPT content in aerial part was found in March for all plants and in the root part in April, except *A. sylvestris* from Enebyberg and Egilstaffr, which were in March. The lowest was between July and September 2009. The highest yields of DPT per plant in the field plants were 22.23 mg in the aerial part in June 2009 and 68.87 mg in the root part in March 2009 (Figure 3C,D). Data are summarized in Table 3.
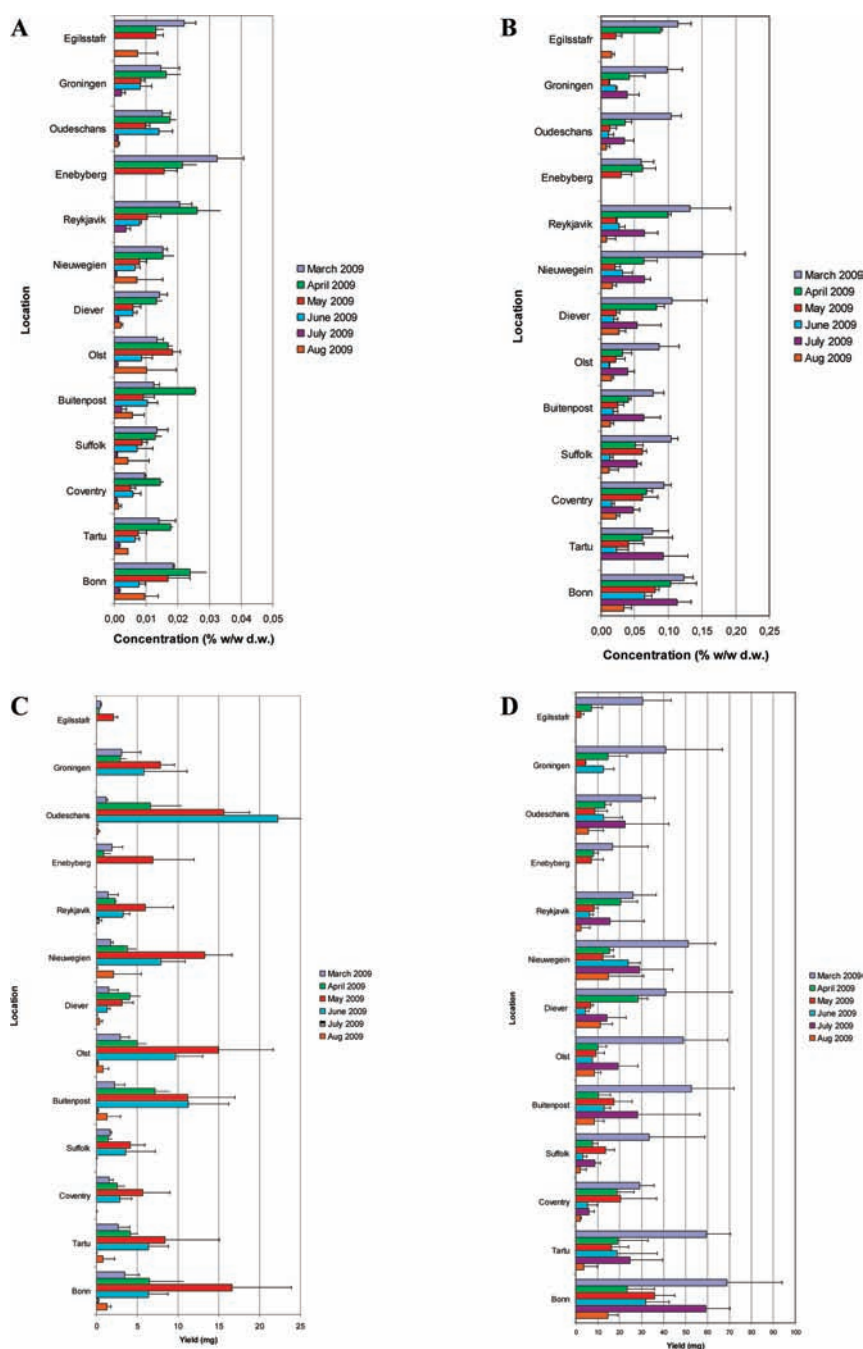
Figure 3. (A) Deoxypodohyllotoxin content in the aerial part of *A. sylvestris* grown in the field; (B) deoxypodohyllotoxin content in the root part of *A. sylvestris* grown in the field; (C) aerial mass of *A. sylvestris* grown in the field; (D) root mass of *A. sylvestris* grown in the field. All DPT contents and yields significantly differ ($p < 0.01$) when indoor and outdoor plants are compared.

DPT Content of the Climate Room Plants (Indoor Plants). Samples were taken during two years following the developmental stages of the life cycle of *A. sylvestris*. The DPT content and yield were measured and compared with outdoor-grown plants of the same seed origin (Figure 4). The DPT content of the climate room plants was up to 0.144% in the roots and up to 0.051% in the aerial part. The highest yield of DPT per plant in the field plants was 2.81 mg in the aerial part and 12.60 mg in the root part in April 2009 (Table 3).

In the first year, the highest DPT content in the roots was found in *A. sylvestris* from Bonn (0.053%) in July 2008. In the

aerial parts the highest content of DPT was found in *A. sylvestris* from Tartu (0.051%) also in July 2008. In the second year, in general the highest DPT contents in both aerial and root parts were found in April 2009 for all plants except *A. sylvestris* from Bonn and Oudechans for aerial parts and *A. sylvestris* from Reykjavik and Oudeschans (Figure 4A,B). At this time the plants started to grow after the cold dormancy period.

Indoor *A. sylvestris* did not flower at the same time as the outdoor plants. All *A. sylvestris* plants from Bonn and Nieuwegein had already started to flower by mid-April 2009, whereas only about 50% of

Table 3. Summary of the Highest and Lowest DPT Content, Biomass, and DPT Yield of *Anthriscus sylvestris* (Figures 3 and 4)

	field		climate room	
	aerial	root	aerial	root
highest DPT content (% w/w, dry wt)	0.033 Enebyberg March 2009	0.151 Nieuwegein March 2009	0.051 Tartu July 2008	0.144 Bonn April 2009
lowest DPT content (% w/w, dry wt)	0.001 Nieuwegein July 2009	0.008 Reykjavik Sept 2009	0.008 Essex June 2009	0.012 Buitenpost Sept 2009
highest mass (dry wt in g)	173.4 Nieuwegein May 2009	108.9 Oudeschans June 2009	7.0 Buitenpost April 2009	16.4 Olst April 2009
lowest mass (dry wt in g)	1.5 Suffolk Aug 2009	8.2 Egilstaffr April 2009	2.0 Bonn June 2008	0.8 Tartu June 2008
highest yield (dry wt in mg) per plant	22.23 Oudeschans June 2009	68.87 Bonn March 2009	2.81 Buitenpost April 2009	12.60 Bonn April 2009
lowest yield (dry wt in mg) per plant	0.01 Coventry July 2009	0.73 Reykjavik Sept 2009	0.12 Essex June 2009	0.32 Coventry Sept 2009

plants from the other origins had started to flower at this time point. The *A. sylvestris* plants from Estonia did not flower at all by mid-April 2009. By end of May 2009, all plants had flowered. In The Netherlands, *A. sylvestris* flowered between April and May.

In the first year, the highest DPT yield in the root part was in August and September 2008 for all plants, but in the aerial parts the highest yield was in July 2008 for all plants. In the second year, the highest DPT yield in the aerial as well as root parts was in April 2009, except the root part of *A. sylvestris* from Groningen (Figure 4C,D).

There is a significant difference when the DPT contents of the field and climate room plants are compared ($p < 0.01$). There is also a significant difference when the total yields of the field and climate room plants are compared ($p < 0.01$).

■ DISCUSSION

A. sylvestris is a biannual plant, meaning that a life cycle takes 2 years. In the first year, the rosettes remain in the vegetative phase and form a basal rosette with a taproot. Vernalization will lead to flowering during the next growing season. After flowering, the seeds are produced and then the life cycle is completed.[34] The biomass of the aerial and root of the field plants reached the maximum in May in the second year for all plants, but the highest DPT content was in March for field plants, in both aerial and root parts. However, only the aerial part of the field plants has the highest yield following the highest biomass in May for all plants except *A. sylvestris* from Oudeschans. The highest yield of the root part for all field plants was in March when the highest DPT content was reached (Table 3).

DPT as the main lignan in *A. sylvestris* was used as a marker to study its metabolic variation over the developmental stages. This is the first study reporting results of climate room experiments covering the entire life cycle of 2 years. In general, the DPT content in the roots was higher than in the aerial parts.

Native versus Cultivated. The first experiment was dedicated to comparison of the DPT content of plants of the native location with that of the same plants grown on the non-native location (Buitenpost). There were significant differences ($p < 0.05$) when the DPT contents in the root parts of all plants except *A. sylvestris* from Enebyberg and Coventry were compared (Figure 2). The DPT content in the aerial part differed moderately over time. Meanwhile, the DPT content in the roots decreased except in *A. sylvestris* from Coventry, U.K. It seems that the *A. sylvestris* from Coventry adapted easily to the Dutch soil. This is reflected in a more or less equal DPT content (0.007% in aerial native compared to 0.005% in aerial cultivation; 0.064% in root native compared to 0.062% in root cultivation). The DPT content of the *A. sylvestris* from Bonn, Germany, was moderately higher (significantly different from the DPT concentration in the root part, $p < 0.05$) compared to others (0.025% in aerial native compared to 0.017% in aerial cultivation; 0.146% in root native compared to 0.081% in root cultivation). On the basis of these results, *A. sylvestris* from Coventry and Bonn were chosen as candidates for further cultivation. The DPT content in roots from the cultivated plants from Groningen was 7.5-fold decreased (0.087% in roots native compared to 0.012% in roots cultivation). This high-producing plant at its native location became a low-producing one when cultivated in a foreign environment.

8136

dx.doi.org/10.1021/jf200177q |*J. Agric. Food Chem.* 2011, 59, 8132–8139
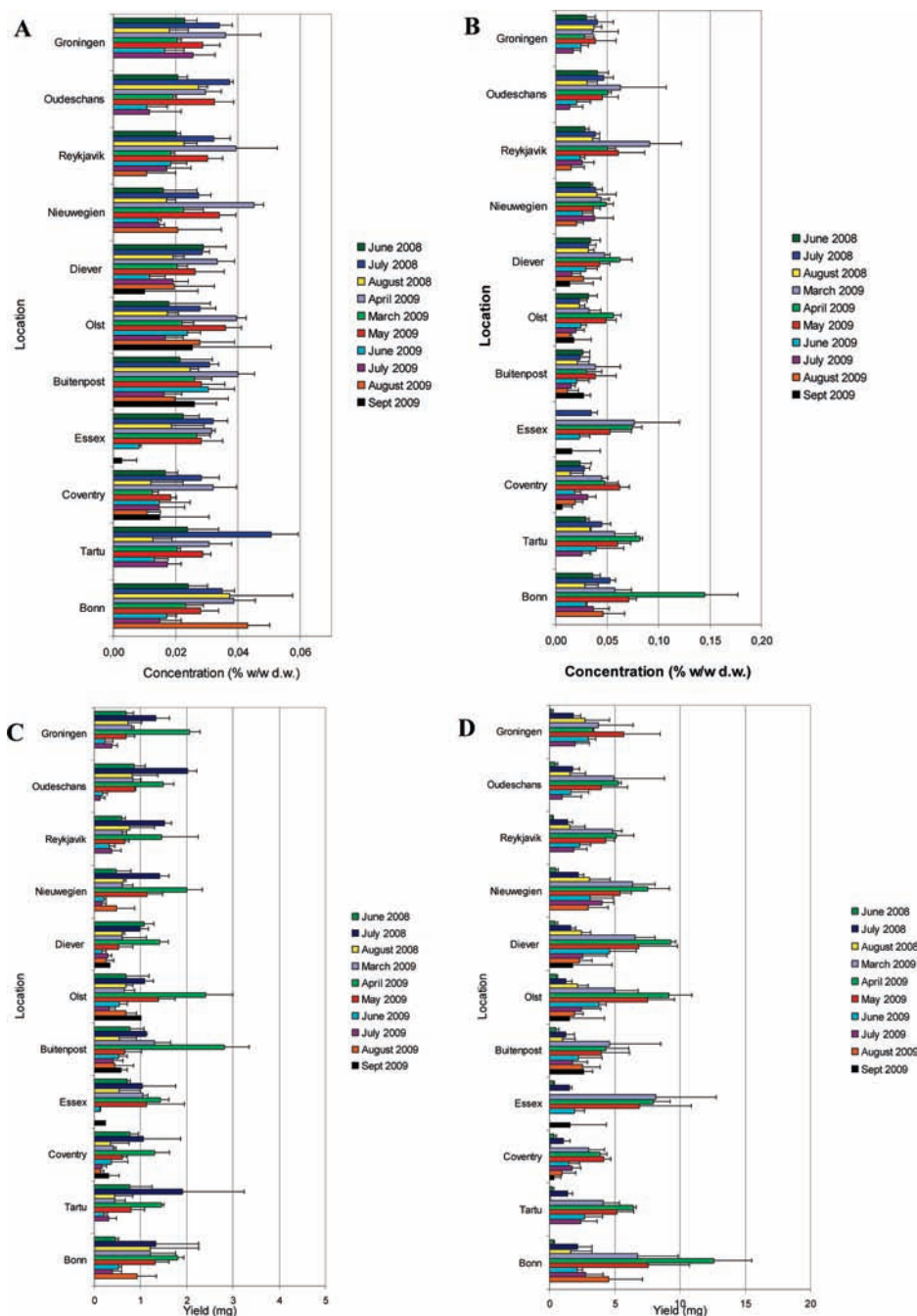
Figure 4. (A) Deoxypodohyllotoxin content in the aerial part of *A. sylvestris* grown in the climate room; (B) deoxypodohyllotoxin content in the root part of *A. sylvestris* grown in the climate room; (C) aerial mass of *A. sylvestris* grown in the climate room; (D) root mass of *A. sylvestris* grown in the climate room. All DPT contents and yields significantly differ ($p < 0.01$) when indoor and outdoor plants are compared.

Interestingly, the DPT content in the root parts of *Anthriscus* from the local area of the botanical garden (Buitenpost) also decreased 5-fold (0.119% in root native compared to 0.024% in root cultivation). In general, the decrease of DPT content in the cultivated root parts varied with a factor between 7.5 and 1 compared to the native plants (0.087% in roots native compared to 0.012% in root cultivation in Groningen *Anthriscus*, 0.064% in roots native compared to 0.062% in root cultivation in Coventry plants). These results clearly show that environmental factors play an important role in the DPT content in *Anthriscus*.

Statistical analysis showed that there is a significant negative correlation among the DPT content, rainfall, temperature, and duration of sun. In both the aerial and root parts, the DPT content is expected to increase with decreasing temperature and duration of sun. The DPT in the aerial part is also influenced by rainfall. The DPT in the aerial part is also expected to increase with decreasing rainfall. There is also a positive significant correlation between the DPT content in the root and aerial parts, which means that the DPT in the aerial part increases with increasing DPT in the root.[35]

This correlation could explain the significant differences ($p < 0.05$) found in DPT content of the root part of all plants

(except *A. sylvestris* from Enebyberg and Coventry) from The Netherlands in 2007 and the Dutch cultivated plants in 2009. These plants originated from Buitenpost, Olst, Groningen, Diever, and Nieuwegein. The DPT content of the cultivated plants became reduced up to 7.5 times in the root parts and up to 3 times in the aerial parts. The DPT content in the root would decrease when the duration of sun and the temperature were increased. In May 2007 the temperature was 1.7 °C higher and there was 38 h less sun as compared to May 2009. This may have caused the DPT content in the root to decrease as shown in Figure 2, whereas in the aerial part, the DPT content would decrease when there were less sun, rainfall, and temperature. In 2007, the weather in The Netherlands was unusual as there was no rain in March 2007, but there was 73 mm more rain in May 2007 compared to May 2009. The more abundant rainfall and higher temperature contributed to the decrease of the DPT in the aerial part; however, the less duration of sun contributed to the increase of the DPT in the aerial part. This might explain why the DPT content was reduced only up to 3 times in the aerial compared to up to 7.5 times in the root part.

Outdoor versus Indoor. The second experiment was to grow all plants in the same field, under the same environmental conditions. In parallel, we also grew the plants under controlled conditions in the climate room. The *A. sylvestris* grown in the field (outdoor) received influences from abiotic and biotic stress, such as temperature, light, drought, wind, and insects, and had more space to grow compared to the plants in the climate room. For experiments of controlled cultivation in a climate room we expect that differences found are mainly to be ascribed to genetic factors, as growing conditions are standardized. Furthermore, it is important to identify rate-limiting ecological factors for improved cultivation either outdoors or indoors.

In the climate room experiment, there was a decrease of DPT production during the vegetative period in the first year (August—February) and also after the plants had flowered (second year). This biosynthetic profile was also observed in *Podophyllum hexandrum*. When the plant gets older, the podophyllotoxin content in the rhizome also decreases.[36] The DPT content in *A. sylvestris* increased again in the spring (March—April, second year). Interestingly, the DPT patterns over the entire period in the climate room were in general similar for all plants (Figure 4).

The DPT profiles of both the indoor and outdoor plants were comparable; however, there were differences in the mass of the aerial and root parts, which influenced the yield, and also in the flowering time of the indoor plants. There is a significant difference ($p < 0.01$) either of the DPT contents or the DPT yields between indoor and outdoor plants. The differences found in DPT production between indoor and outdoor plants were comparable to those from a previous study by our group[31] and others.[37] The interindividual variability of the DPT content in the root parts of the outdoors plants was higher than of the indoor plants (e.g., 0.151—0.007% versus 0.144—0.012%; see Table 3). In contrast, the interindividual variability of the DPT content in the aerial part of the indoor plants was higher than of the outdoor plants (e.g., 0.051—0.008% versus 0.033—0.001%; see Table 3). Previous research showed that the indoor plants contained higher DPT contents in aerial parts than in the roots.[31] We now confirm these results as several indoor plants from Estonia (0.051% in aerial, 0.045% in root), Buitenpost (0.031% in aerial, 0.024% in root), and Olst (0.028% in aerial, 0.023% in root), all sampled in July 2008, had higher DPT contents in the aerial part compared to the roots.

The differences found in the flowering time of the indoor plants point toward variations even though the plants were grown under identical conditions. These variations might point to differences in genotype at different geographical locations. The similarity in DPT contents observed under controlled conditions suggested that these genetic differences, if any, do not influence the DPT production. However, the significant differences in DPT content and yield between outdoor and indoor plants suggest that there might be environmental factors that play a role in DPT production and yield.

The root mass of the outdoor plants was up to 8 times higher than that of the indoor plants, and the aerial mass was up to 30 times higher than that of the indoor plants. This influenced the DPT yield. The DPT yield of the outdoor plants was up to 8 times higher in the aerial part and up to 5 times higher in the root part compared with the indoor plants. This variation in the root and aerial mass of the indoor plants was also observed in the previous study.[31]

In summary, these results show the DPT content over time for both indoor and outdoor plants. The best time to harvest plants for DPT isolation is when the plants reach the optimal DPT production together with an adequate biomass. For indoor plants this was in April (second year) in both aerial and root parts. For outdoor plants this was in March (second year) for the root part and in June (second year) for the aerial part. *A. sylvestris* from Bonn showed constant production of DPT in the field over the season and may be a suitable candidate for ongoing cultivation and breeding for production purposes and commercial isolation of DPT (Figure 3). Another candidate is *A. sylvestris* from Coventry, which showed a similar DPT content when cultivated elsewhere (Figure 2), but further work to optimize growing conditions must be carried out.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: +49-231-7557487. Fax: +49-231-7557489. E-mail: oliver.kayser@bci.tu-dortmund.de.

■ REFERENCES

(1) Jeong, G.; Kwon, O.; Park, B.; Oh, S.; Ahn, K.; Chang, M.; Oh, W.; Kim, J.; Min, B.; Kim, Y.; Lee, H. Lignans and coumarins from the roots of *Anthriscus sylvestris* and their increase of caspase-3 activity in HL-60 cells. *Biol. Pharm. Bull.* **2007**, *30*, 1340–1343.

(2) Magnusson, S. H. NOBANIS — invasive alien species fact sheet *Anthriscus sylvestris*, 2006; http://www.nobanis.org/files/factsheets/Anthriscus_sylvestris.pdf (accessed May 3, 2011).

(3) Hulten, E.; Fries, M. *Atlas of North European Vascular Plants North of the Tropic of Cancer*; Koeltz Scientific Books: Konigstein, Germany, 1986; Vol. 2.

8138

dx.doi.org/10.1021/jf200177q |*J. Agric. Food Chem.* 2011, 59, 8132–8139

(4) Yong, Y.; Shin, S. Y.; Lee, Y. H.; Lim, Y. Antitumor activity of deoxypodophyllotoxin isolated from *Anthriscus sylvestris*: induction of G2/M cell cycle arrest and caspase-dependent apoptosis. *Bioorg. Med. Chem. Let.* **2009**, *19*, 4367–4371.

(5) Wong, S.; Tsui, S.; Kwan, S.; Su, X.; Lin, R. Identification and characterization of *Podophyllum emodi* by API-LC/MS/MS. *J. Mass Spectrom.* **2000**, *35*, 1246–1251.

(6) Koulman, A.; Kubbinga, M. E.; Batterman, S.; Woerdenbag, H. J.; Pras, N.; Woolley, J. G.; Quax, W. J. A phytochemical study of lignans in whole plants and cell suspension cultures of *Anthriscus sylvestris*. *Planta Med.* **2003**, *69*, 733–738.

(7) Koulman, A.; Bos, R.; Medarde, M.; Pras, N.; Quax, W. J. A fast and simple GC MS method for lignan profiling in *Anthriscus sylvestris* and biosynthetically related plant species. *Planta Med.* **2001**, *67*, 858–862.

(8) Kozawa, M.; Baba, K.; Matsuyama, Y.; Kido, T.; Sakai, M.; Takemoto, T. Components of the root of *Anthriscus sylvestris* Hoffm. II. Insecticidal acitivity. *Chem. Pharm. Bull.* **1982**, *30*, 2885–2888.

(9) Charlton, J. L. Antiviral activity of lignans. *J. Nat. Prod.* **1998**, *61*, 1447–1451.

(10) Jin, M.; Moon, T. C.; Quan, Z.; Lee, E.; Kim, Y. K.; Yang, J. H.; Suh, S.; Jeong, T. C.; Lee, S. H.; Kim, C.; Chang, H. W. The naturally occurring flavolignan, deoxypodophyllotoxin, inhibits lipopolysaccharide-induced iNOS expression through the NF-κB activation in RAW264.7 macrophage cells. *Biol. Pharm. Bull.* **2008**, *31*, 1312–1315.

(11) Jin, M.; Lee, E.; Yang, J. H.; Lu, Y.; Kang, S.; Chang, Y. C.; Lee, S. H.; Suh, S. J.; Kim, C. H.; Chang, H. W. Deoxypodophyllotoxin inhibits the expression of intercellular adhesion molecule-1 induced by tumor necrosis factor-α; in murine lung epithelial cells. *Biol. Pharm. Bull.* **2010**, *33*, 1–5.

(12) Chen, J. J.; Chang, Y. L.; Teng, C. M.; Chen, I. S. Anti-platelet aggregation alkaloids and lignans from *Hernandia nymphaeifolia*. *Planta Med.* **2000**, *66*, 251–256.

(13) Gordaliza, M.; Castro, M. A.; García-Grávalos, M. D.; Ruiz, P.; Corral, J. M. M. D.; Feliciano, A. S. Antineoplastic and antiviral activities of podophyllotoxin related lignans. *Arch. Pharm.* **1994**, *327*, 175–179.

(14) Lee, S. H.; Son, M. J.; Ju, H. K.; Lin, C. X.; Moon, T. C.; Choi, H. G.; Son, J. K.; Chang, H. W. Dual inhibition of cyclooxygenases-2 and 5-lipoxygenase by deoxypodophyllotoxin in mouse bone marrow-derived mast cells. *Biol. Pharm. Bull.* **2004**, *27*, 786–788.

(15) Sudo, K.; Konno, K.; Shigeta, S.; Yokota, T. Inhibitory effects of podophyllotoxin derivatives on herpes simplex virus replication. *Antivir. Chem. Chemother.* **1998**, *9*, 263–267.

(16) Ikeda, R.; Nagao, T.; Okabe, H.; Nakano, Y.; Matsunaga, H.; Katano, M.; Mori, M. Antiproliferative constituents in umbelliferae plants. III. Constituents in the root and the ground part of *Anthriscus sylvestris* Hoffm. *Chem. Pharm. Bull.* **1998**, *46*, 871–874.

(17) Shin, S. Y.; Yong, Y.; Kim, C. G.; Lee, Y. H.; Lim, Y. Deoxypodophyllotoxin induces G2/M cell cycle arrest and apoptosis in HeLa cells. *Cancer Lett.* **2010**, *287*, 231–239.

(18) Choi, H.; Lee, J.; Shin, H.; Lee, B.; Chang, I.; Hwang, J. Deoxypodophyllotoxin reduces skin pigmentation of brown guinea pigs. *Planta Med.* **2004**, *70*, 378–380.

(19) Suh, S.; Kim, J.; Jin, U.; Choi, H.; Chang, Y.; Lee, Y.; Kim, S.; Lee, I.; Moon, T.; Chang, H.; Kim, C. Deoxypodophyllotoxin, flavolignan, from *Anthriscus sylvestris* Hoffm. inhibits migration and MMP-9 via MAPK pathways in TNF-α-induced HASMC. *Vasc. Pharm.* **2009**, *51*, 13–20.

(20) Lin, C. X.; Lee, E.; Jin, M. H.; Yook, J.; Quan, Z.; Ha, K.; Moon, T. C.; Kim, M. J.; Kim, K. J.; Lee, S. H.; Chang, H. W. Deoxypodophyllotoxin (DPT) inhibits eosinophil recruitment into the airway and Th2 cytokine expression in an OVA-induced lung inflammation. *Planta Med.* **2006**, *72*, 786–791.

(21) Dall'Acqua, S.; Giorgetti, M.; Cervellati, R.; Innocenti, G. Deoxypodophyllotoxin content and antioxidant activity of aerial parts of *Anthriscus sylvestris* Hoffm. *Z. Naturforsch. C* **2006**, *61*, 658–662.

(22) Rong, G.; Congfen, G.; Xuan, T.; Xiangyang, Y.; Xudong, D.; Hang, X.; Xing, Z. Insecticidal activity of deoxypodophyllotoxin, isolated from *Juniperus sabina* L., and related lignans against larvae of *Pieris rapae* L. *Pest Manag. Sci.* **2004**, *60*, 1131–1136.

(23) Xu, H.; Wang, J. J. Natural products-based insecticidal agents 5. Design, semisynthesis and insecticidal activity of novel 4′-substituted benzenesulfonate derivatives of 4-deoxypodophyllotoxin against *Mythimna separata* Walker *in vivo*. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2500–2502.

(24) Ayres, D. C.; Loike, J. D. Lignans: chemical, biological and clinical properties. In *Chemistry and Pharmacology of Natural Products*; Cambridge University Press: Cambridge, U.K., 1990.

(25) Federolf, K.; Alfermann, A. W.; Fuss, E. Aryltetralin-lignan formation in two different cell suspension cultures of *Linum album*: deoxypodophyllotoxin 6-hydroxylase, a key enzyme for the formation of 6-methoxypodophyllotoxin. *Phytochemistry* **2007**, *68*, 1397–1406.

(26) Jackson, D. E.; Dewick, P. M. Aryltetralin lignans from *Podophyllum hexandrum* and *Podophyllum peltatum*. *Phytochemistry* **1984**, *23*, 1147–1152.

(27) Kondo, K.; Ogura, M.; Midorikawa, Y.; Kozawa, M.; Tsujibo, H.; Baba, K.; Inamori, Y. Conversion of deoxypodophyllotoxin to podophyllotoxin-related compounds by microbes. *Agric. Biol. Chem.* **1989**, *53*, 777–782.

(28) Choudhary, D. K.; Kaul, B. L.; Khan, S. Cultivation and conservation of *Podophyllum hexandrum*—an overview. *J. Med. Aromat. Plant Sci.* **1998**, *20*, 1071–1073.

(29) Nayar, M. P.; Sastry, A. P. K. *Red Data Book of Indian Plants*; Calcutta, 1990.

(30) Giri, A.; Lakshmi Narasu, M. Production of podophyllotoxin from *Podophyllum hexandrum*: a potential natural product for clinically useful anticancer drugs. *Cytotechnology* **2000**, *34*, 17–26.

(31) Koulman, A.; Batterman, S.; van Putten, F. M. S.; Bos, R.; Quax, W. J. Lignan profiles of indoor-cultivated *Anthriscus sylvestris*. *Planta Med.* **2003**, *69*, 959–961.

(32) Koulman, A.; Hendrawati, O.; Batterman, S.; Van Putten, F. M. S.; Bos, R.; Kayser, O. The seasonal variations of lignan profiles in *Anthriscus sylvestris* (L.) Hoffm. *Planta Med.* **2007**, *73*, P_112.

(33) Vasilev, N. P.; Julsing, M. K.; Koulman, A.; Clarkson, C.; Woerdenbag, H. J.; Ionkova, I.; Bos, R.; Jaroszewski, J. W.; Kayser, O.; Quax, W. J. Bioconversion of deoxypodophyllotoxin into epipodophyllotoxin in *E. coli* using human cytochrome P450 3A4. *J. Biotechnol.* **2006**, *126*, 383–393.

(34) Van Mierlo, J. E. M.; Van Groenendael, J. M. A population dynamic approach to the control of *Anthriscus sylvestris* (L.) Hoffm. *J. Appl. Ecol.* **1991**, *28*, 128–139.

(35) Field, A. *Discovering Statistics Using SPSS*, 2nd ed.; SAGE Publications: London, U.K., 2005; pp 125−129.

(36) Pandey, H.; Nandi, S.; Kumar, A.; Palni, U.; Palni, L. Podophyllotoxin content in *Podophyllum hexandrum* Royle plants of known age of seed origin and grown at a lower altitude. *Acta Physiol. Planta* **2007**, *29*, 121–126.

(37) Robinson, A. R.; Ukrainetz, N. K.; Kang, K.; Mansfield, S. D. Metabolite profiling of Douglas-fir (*Pseudotsuga menziesii*) field trials reveals strong environmental and weak genetic variation. *New Phytol.* **2007**, *174*, 762–773.