Distribution of Hydrolysable Tannins in the Foliage of Finnish Birch Species

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On the basis of qualitative and quantitative analysis with liquid chromatography – electrospray ionisation mass spectrometry, the foliage of dwarf birch (*Betula nana* L.), silver birch (*B. pendula* Roth) and mountain birch (*B. pubescens* ssp. c*zerepanovii* (Orlova) Hämet-Ahti) were found, for the first time, to contain the same individual HTs that were described earlier for white birch (*B. pubescens* Ehrh.). In addition, one previously unidentified ellagitannin was preliminarily identified from the leaves of white and mountain birches, being totally absent from the foliage of the other two species. There were large variations in the contents of HTs between species. Seasonal variation affected significantly the contents of some individual HTs within species, and these changes were mainly in accordance with the biosynthetic pathway of HTs. All species converted galloylglucoses (GGs) into ellagitannins (ETs), dwarf birch being the only one that's efficient ET synthesis resulted in seasonally increased contents of ETs and thereof total HTs as well. The presence of insoluble ETs as well as the absence of insoluble GGs was confirmed in all four birch species for the first time. Furthermore, the amounts of insoluble ETs per one birch leaf were found to accumulate during the growing season. These findings complemented our knowledge of the biosynthetic pathway of birch leaf HTs: from soluble GGs via soluble ETs into insoluble ETs. The possible role of HTs in the herbivore defence of these species is discussed.

Introduction

Hydrolysable tannins (HTs) are a heterogeneous group of polyphenolic compounds that have been assumed to play an important role in plant-herbivore interactions. They have been shown to possess a wide variety of chemical structures, which are not necessarily equal in their biological activities. For instance the protein precipitation capacity, one of the most commonly used measures for the biological activity of tannins in general, has been shown to vary greatly between individual HTs (Kilkowski and Gross, 1999). The hydrolysis products of individual tannins and their metabolism within the insect digestive tract have been shown to differ as well, resulting consequently to negative (Klocke *et al.*, 1986), negligible or even positive effects (Bernays *et al.*, 1989) for the insect. In ecological studies HTs have been usually quantified simply as a total content of their subgroups, galloylglucoses (GGs) or ellagitannins (ETs). By measuring values that contain a pool of compounds with several modes of action, a lot of ecological as well as chemotaxonomical information about the distribution of biologically active and non-active tannins from different plants, and towards different herbivores, may have been lost. To circumvent these problems, we have used high-performance liquid chromatography (HPLC) connected with electrospray ionisation mass spectrometer (ESI-MS) for both qualitative and quantitative analysis of HTs (Salminen *et al.*, 1999, 2001; Rauha *et al.*, 2001). The quantitative HPLC-ESI-MS method that is based on ion trace analysis of individual HTs, is able to separate and detect all birch leaf HTs based on their known molecular weights. Because of this high selectivity, the use of ESI-MS with HPLC excludes the possibility of false detection even in complex samples where HTs would be only minor compounds overlapped by other phenolics.

Although there have been several studies on birch leaf phenolics (Ossipov *et al.*, 1995, 1996, 1997, 2001; Keinänen and Julkunen-Tiitto, 1998; Lavola *et al.*, 2000; Graglia *et al.*, 2001), detailed information on the content of HTs has been published from leaves of white birch (*Betula pubescens*) only (Salminen *et al.*, 1999, 2001). Never-

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theless, the high total content of GGs has been shown to reduce the suitability of mountain birch (*B. pubescens* ssp. c*zerepanovii*) leaves for larvae of the geometrid moth *Epirrita autumnata*, potentially the most destructive insect pest of birch (Kause *et al*., 1999; Ossipov *et al*., 2001). Furthermore, depending on the nature and level of HTs in the foliage, their biological effects are shown to be either defensive (Kause *et al*., 1999) or even increasing the fitness of the herbivore (Salminen and Lempa, submitted). Therefore the knowledge of the distribution of individual HTs as well as their levels and seasonal variation in the leaves of different birch species would contribute to an understanding of a possible herbivore defence of these species.

The aim of this study was to investigate the distribution and contents of hydrolysable tannins in four Finnish birch species, *B. pubescens*, *B. nana*, *B. pendula* and *B. pubescens* ssp. c*zerepanovii*. We also wanted to check if the seasonal variations in the content of individual HTs in all birch species would be in accordance with the biosynthetic pathways described earlier for GGs and ETs in white birch (Salminen *et al*., 2001). Furthermore, the earlier proposed transformation of soluble HTs into insoluble cell-wall-bound constituents (Ossipov *et al*., 1997, 2001) needed to be clarified. The possible role of HTs in the herbivore defence of these species is discussed as well.

Materials and Methods

Plant material

Leaves of white birch (*B. pubescens* Ehrh.) and silver birch (*B. pendula* Roth) were sampled in the Botanical garden of the University of Turku, SW-Finland. Dwarf birch (*B. nana* L.) samples were collected in a swamp near Turku, SW-Finland, while leaves of mountain birch (*B. pubescens* ssp. c*zerepanovii* (Orlova) Hämet-Ahti) were sampled near the Kevo Subarctic Research Station of University of Turku, in Northern Finland. All the experimental specimens were within the natural range of the respective species. To get samples that were approximately at the same phenological stage, leaf sampling in Kevo was done later than in Turku. Samples from ten individual trees per species were taken twice during the growing season of 1999. Samples of 10–15 short shoot leaves per tree were collected between 9 and 12 a.m. haphazardly from branches. The leaves were clipped from the petioles and placed in sealed plastic vials, which were enclosed in an insulated box filled with ice and transported immediately to a laboratory freezer.

Sample preparation

The freeze-dried birch leaves (300 mg dry wt) were extracted (3 × 1 h) with 70% aq. Me_2CO (3 × 10 ml) in a planar shaker. After removal of Me_2CO at room temp., the aq. extract was freeze-dried and dissolved in 6 ml water. These aq. samples were filtered through 0.45 μm PTFE filters and kept frozen at −20 °C until analysed with HPLC-ESI-MS. The insoluble residue of birch leaves that was not extractable with 70% aq. Me_2CO was lyophilised, weighed and stored at −20 °C until used in the analysis of insoluble ETs.

Analysis of hydrolysable tannins with HPLC-ESI-MS

HPLC-ESI-MS analysis of birch leaf extracts was performed using a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ion-spray (pneumatically assisted electrospray) interface. The HPLC system consisted of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT, USA) connected to a Series 200 autosampler. The eluate passed through a Merck Superspher 100 RP-18 column (75 × 4 mm i.d., 4 μm), and the UV trace was recorded with a 785A UV/VIS detector at 280 nm. After UV detection, part of the eluate was split off and introduced into the ESI-MS system. Chromatographic and ESI-MS conditions were as described in a previous paper (Salminen *et al*., 1999). Birch leaf extracts were qualitatively analysed with negative ion HPLC-ESI-MS and HTs were identified on the basis of their $[M-H]^-$ values and retention times as in Salminen *et al.* (1999, 2001). The identified HTs were quantified using ion trace analysis of deprotonated molecules ($[M-H]^-$) of individual tannins and calibration curves of the appropriate HTs as previously shown (Salminen *et al.*, 1999, 2001). In addition, the new pedunculagin derivative isolated in this study was quantified as pedunculagin equivalents. Because of the low content of HTs in the leaves

of silver birch, all silver birch samples were concentrated by a factor 2.5 before HPLC-ESI-MS analysis.

Analysis of insoluble ellagitannins

Insoluble ETs of birch leaves were quantified with our modification of the method described by Peng *et al.* (1991). 50 mg of lyophilised 70% Me_2CO -insoluble residue of birch leaves was hydrolysed for 3 h with 5 ml MeOH/6M HCl (9:1) at 120 °C to liberate the ellagic acid that was bound to insoluble ETs as hexahydroxydiphenoyl (HHDP) residues. After cooling to room temperature, the hydrolysate was filtered through 0.45 μm PTFE filters and analysed directly with HPLC at 258 nm. The HPLC system (Merck-Hitachi, Tokyo, Japan) consisted of a pump L-7100, a UV detector L-7400, a programmable autosampler L-7200, and an interface D-7000. The column was Superspher 100 RP-18 (75 × 4 mm i.d., 4 μm, Merck, Germany) and eluents 0.05M H_3PO_4 (A) and CH_3CN (B). The elution profile was: 0–3 min, 98% A (isocratic); 3–22 min, 2–20% B in A (linear gradient); 22–30 min, 20–30% B in A (linear gradient) at a flow rate 1 ml min^{-1}. Ellagic acid was detected on the basis of its retention time compared to the authentic standard. The identity of the ellagic acid peak was confirmed with its UV and mass spectra from two randomly chosen samples of each birch species. The content of insoluble ETs was quantified as mg ellagic acid g^{-1} dry weight of birch leaves.

Isolation and preliminary identification of the new ellagitannin

Freeze-dried white birch leaves (50 g dry wt) were extracted with 70% aq. Me_2CO (containing 0.1% ascorbic acid to prevent oxidation) and fractionated on a Sephadex LH-20 column (40 × 2.5 cm i.d.) into seven fractions (frs 1–7), as previously described (Salminen *et al.*, 1999). On the basis of HPLC-DAD analysis (UV detector L-7400 was replaced with diode array detector L-7455) of the fractions, the isolated ET (purity 30% at 280 nm) was detected in fr 3 (eluted with 50% MeOH). Fr 3 was fractionated further on a Sephadex LH-20 column with a step-gradient of more dilute MeOH (from 10% to 35%) and 29 fractions of 100 ml were collected. The isolated ET was detected in frs 20–22, which were combined (purity 55%) and fractionated further by chromatography on a Merck LiChroprep RP-18 column (44 × 3.7 cm i.d., 40–63 μm) using step-gradients of CH_3CN (from 5% to 60%) in H_2O. The fractions containing the ET (purity 81%) were combined, reduced to aq. phase and extracted with ethylacetate (4 × 50 ml). The aq. layer that contained the ET (purity 90%) was lyophilised and weighed (40 mg).

The structure of the ET was studied with HPLC-DAD, HPLC-ESI-MS and ^{1}H NMR as previously shown (Salminen *et al.*, 1999, 2001). In addition, the ET was hydrolysed in water at 100 °C for 3 h, in MeOH/6 M HCl (9:1) at 120 °C for 3 h, and refluxed in MeOH for 3 h. Reaction products were analysed with HPLC-DAD and HPLC-ESI-MS.

Statistical methods

Since leaves were sampled twice during the growing season, the two successive samples from an individual tree were not independent in a statistical sense. Therefore, the appropriate statistical method, paired two-sample Student's t-test, was used to analyse differences between the two dates (Scheiner and Gurevitch, 1993).

Results and Discussion

Preliminary identification of the new ellagitannin

The UV spectrum of the isolated compound was characteristic for an ET having at least one HHDP-group (Salminen *et al.*, 1999). The compound produced two peaks in HPLC (9.7 and 14.9 min), which is a typical property for an ET that has an ungalloylated anomeric centre, i.e. no galloyl groups at C-1 of the glucose (Salminen *et al.*, 2001). The α and β isomers of the anomeric mixture were always present at 50:50 equilibrium in solutions and therefore it was not possible to isolate them permanently from each other. Since ETs like vescalagin or castalagin that have an acyclic glucose core do not form such an equilibrium (Salminen, personal observation), the glucose of the isolated ET could not have been an acyclic one, but a glucopyranose.

The ^{1}H NMR spectrum was complicated because of the presence of both α and β isomers and some impurities that increased within time presumably as a result of oxidation or degradation of

the compound in the D_2O/Me_2CO-d_6 media. However, the proton signals between 4.8 and 5.6 ppm corresponded with those obtained earlier for glucose protons H-1, H-2 and H-3 of 2,3-(*S*)-HHDP-glucose (Lee *et al.*, 1992; Salminen *et al.*, 2001). Both the hydrolysis of the compound in 100 °C water and refluxing in MeOH resulted in formation of 2,3-(*S*)-HHDP-glucose, which was identified on the basis of its retention times (1.6 and 2.8 min, anomeric mixture) and UV and mass spectra (Salminen *et al.*, 1999, 2001). Thus the presence of an HHDP-group at 2,3-position of the glucose was confirmed, leaving only the attachments at positions 4 and 6 of the glucose unclear.

The main *m/z* values for the compound in negative and positive ion HPLC-ESI-MS were 787 and 789, respectively. Thus the molecular weight of the compound was 788. It gave also *m/z* values 769 and 301 in the negative mode, and 771 and 303 in the positive mode corresponding to losses of H_2O and an HHDP-group. Since we have not detected a loss of water from any other birch leaf ET in ESI-MS, it has to be a characteristic for the unusual attachment at 4,6-position of the glucose. This was confirmed with a negative mode product ion scan of *m/z* 769 ($[M-H_2O-H]^-$) that still produced *m/z* values 301 and 481 corresponding to an HHDP-group and an HHDP-glucose, respectively.

Since the compound was present at relatively high levels in leaves of white and mountain birches (Tables II-III), we assumed it to be a modification of pedunculagin, which was otherwise the main ET in all birch species studied. The closely related mass value (788) to that of pedunculagin (784) indicated a difference of 4 atomic mass units in the 4,6-attachment of the compound, since otherwise the structure of pedunculagin is similar to the isolated ET. That difference could be explained by unusual reduction of both C=O groups of 4,6-HHDP into CHOH, but since this could not be confirmed with ^{1}H NMR partly because of tannin degradation, we identified this new ET simply as a pedunculagin derivative.

Table I. The contents of individual hydrolysable tannins and insoluble ellagitannins in the foliage of silver birch (*Betula pendula*).

Compound	Content mg/g dw		Significance of differences[a]
	June 1	June 16	
1-Galloylglucose	n.s.	n.s.	
1,6-Digalloylglucose	n.s.	n.s.	
1,2,6-Trigalloylglucose	n.s.	n.s.	
1,2,3,6-Tetragalloylglucose	n.s.	n.s.	
1,2,3,4,6-Pentagalloylglucose	0.11 ± 0.01	0.08 ± 0.01	*
Total galloylglucoses	0.11 ± 0.01	0.08 ± 0.01	*
Pedunculagin derivative	n.d.	n.d.	
Pedunculagin	0.30 ± 0.04	0.21 ± 0.03	***
Tellimagrandin I	0.19 ± 0.02	0.09 ± 0.01	***
Casuarictin	0.10 ± 0.02	0.02 ± 0.01	***
Potentillin	0.10 ± 0.01	0.03 ± 0.01	***
Tellimagrandin II	0.08 ± 0.01	0.07 ± 0.01	*
1,2,3-Trigalloyl-4,6-(*S*)-HHDP-α-D-glucose	0.11 ± 0.01	0.08 ± 0.01	
Isostrictinin	0.02 ± 0.01	n.s.	*
2,3-(*S*)-HHDP-glucose	n.s.	n.s.	
Total ellagitannins	0.90 ± 0.10	0.50 ± 0.04	***
Total hydrolysable tannins	1.01 ± 0.11	0.58 ± 0.04	***
Insoluble ellagitannins	0.41 ± 0.02	0.33 ± 0.02	*

Values are mean contents of ten trees ± SE.
n.s. = not significant.
n.d. = not detected.
[a] * $P<0.05$, *** $P<0.001$.

Table II. The contents of individual hydrolysable tannins and insoluble ellagitannins in the foliage of white birch (*Betula pubescens*).

Compound	Content mg/g dw		Significance of differences[a]
	June 1	June 14	
1-Galloylglucose	0.69 ± 0.17	0.32 ± 0.10	**
1,6-Digalloylglucose	0.44 ± 0.12	0.32 ± 0.11	
1,2,6-Trigalloylglucose	0.36 ± 0.06	0.31 ± 0.08	
1,2,3,6-Tetragalloylglucose	0.32 ± 0.05	0.23 ± 0.04	*
1,2,3,4,6-Pentagalloylglucose	1.07 ± 0.17	0.52 ± 0.12	***
Total galloylglucoses	2.88 ± 0.40	1.70 ± 0.31	***
Pedunculagin derivative	3.00 ± 0.87	2.61 ± 0.75	
Pedunculagin	1.60 ± 0.43	1.93 ± 0.45	
Tellimagrandin I	1.23 ± 0.14	0.96 ± 0.19	
Casuarictin	0.66 ± 0.06	0.36 ± 0.06	**
Potentillin	0.72 ± 0.09	0.47 ± 0.09	
Tellimagrandin II	0.59 ± 0.07	0.23 ± 0.05	***
1,2,3-Trigalloyl-4,6-(*S*)-HHDP-α-D-glucose	0.40 ± 0.05	0.43 ± 0.06	
Isostrictinin	0.15 ± 0.02	0.15 ± 0.03	
2,3-(*S*)-HHDP-glucose	0.09 ± 0.03	0.18 ± 0.05	*
Total ellagitannins	8.44 ± 0.98	7.31 ± 1.23	
Total hydrolysable tannins	11.32 ± 1.28	9.02 ± 1.48	*
Insoluble ellagitannins	0.64 ± 0.07	0.76 ± 0.09	**

Values are mean contents of ten trees ± SE.
[a] * P<0.05, ** P<0.01, *** P<0.001.

Table III. The contents of individual hydrolysable tannins and insoluble ellagitannins in the foliage of mountain birch (*Betula pubescens ssp. czerepanovii*).

Compound	Content mg/g dw		Significance of differences[a]
	June 27	July 27	
1-Galloylglucose	1.77 ± 0.57	1.04 ± 0.32	*
1,6-Digalloylglucose	1.12 ± 0.34	1.27 ± 0.34	
1,2,6-Trigalloylglucose	1.58 ± 0.51	0.96 ± 0.26	*
1,2,3,6-Tetragalloylglucose	0.52 ± 0.30	0.24 ± 0.09	*
1,2,3,4,6-Pentagalloylglucose	1.70 ± 0.90	0.68 ± 0.30	***
Total galloylglucoses	6.68 ± 2.49	4.19 ± 1.11	**
Pedunculagin derivative	5.61 ± 1.59	5.22 ± 1.50	
Pedunculagin	4.09 ± 1.85	4.16 ± 1.71	
Tellimagrandin I	1.66 ± 0.69	0.96 ± 0.50	**
Casuarictin	0.59 ± 0.25	0.43 ± 0.29	*
Potentillin	0.29 ± 0.13	0.11 ± 0.08	**
Tellimagrandin II	0.36 ± 0.13	0.24 ± 0.12	***
1,2,3-Trigalloyl-4,6-(*S*)-HHDP-α-D-glucose	0.49 ± 0.22	0.43 ± 0.22	**
Isostrictinin	0.09 ± 0.06	0.10 ± 0.07	
2,3-(*S*)-HHDP-glucose	0.16 ± 0.05	0.47 ± 0.15	*
Total ellagitannins	13.34 ± 4.27	12.11 ± 4.08	
Total hydrolysable tannins	20.02 ± 6.75	16.29 ± 5.10	*
Insoluble ellagitannins	0.63 ± 0.08	0.64 ± 0.12	

Values are mean contents of ten trees ± SE.
[a] * P<0.05, ** P<0.01, *** P<0.001.

Table IV. The contents of individual hydrolysable tannins and insoluble ellagitannins in the foliage of dwarf birch (*Betula nana*).

Compound	Content mg/g dw		Significance of differences[a]
	June 1	June 16	
1-Galloylglucose	1.73 ± 0.51	1.29 ± 0.30	
1,6-Digalloylglucose	1.97 ± 0.51	2.06 ± 0.37	
1,2,6-Trigalloylglucose	1.19 ± 0.33	1.44 ± 0.42	
1,2,3,6-Tetragalloylglucose	0.77 ± 0.27	0.72 ± 0.24	
1,2,3,4,6-Pentagalloylglucose	0.55 ± 0.14	0.37 ± 0.10	
Total galloylglucoses	6.21 ± 1.71	5.87 ± 1.29	
Pedunculagin derivative	n.d.	n.d.	
Pedunculagin	7.41 ± 2.44	14.67 ± 3.68	**
Tellimagrandin I	4.33 ± 1.65	5.35 ± 2.00	
Casuarictin	1.96 ± 0.55	4.41 ± 1.23	**
Potentillin	0.88 ± 0.20	0.45 ± 0.15	**
Tellimagrandin II	0.84 ± 0.24	1.15 ± 0.35	
1,2,3-Trigalloyl-4,6-(*S*)-HHDP-α-D-glucose	0.24 ± 0.10	0.19 ± 0.03	
Isostrictinin	0.38 ± 0.08	0.52 ± 0.15	
2,3-(*S*)-HHDP-glucose	0.06 ± 0.02	0.17 ± 0.05	*
Total ellagitannins	16.10 ± 4.75	26.91 ± 6.96	*
Total hydrolysable tannins	22.31 ± 6.31	32.78 ± 8.19	*
Insoluble ellagitannins	0.42 ± 0.06	0.50 ± 0.06	

Values are mean contents of ten trees ± SE.
n.d. = not detected.
[a] * $P<0.05$, ** $P<0.01$.

Contents and seasonal variations of galloylglucoses

On the basis of HPLC-ESI-MS analysis, the foliage of *B. nana*, *B. pendula* and *B. pubescens* ssp. c*zerepanovii* were found, for the first time, to contain the same individual GGs that were described earlier for *B. pubescens* (Salminen *et al*., 2001). The characteristic trend for the content of GGs, i.e. seasonal decline (Salminen *et al*., 2001), was clearest among the white and mountain birches (see Tables I-IV). In these species the content of the end product of the GG-pathway, i.e. pentagalloylglucose (PGG), decreased most significantly ($P<0.001$) of the GGs, possibly due to its transformation into ETs (Salminen *et al*., 2001). PGG was one of the main GGs in all species, except in dwarf birch, of which active ET synthesis produced temporally increasing amounts of ETs so effectively that PGG could not accumulate. Interestingly, PGG was the only silver birch GG that was found in significant levels, again indicating of its late position in the GG-pathway and of the lack of active synthesis of ETs in silver birch.

Contents and seasonal variations of ellagitannins

Individual ETs, or ETs in general, were detected for the first time from leaves of *B. nana*, *B. pendula* and *B. pubescens* ssp. c*zerepanovii*. The composition of ETs in all species was mainly the same that was described earlier for *B. pubescens* (Salminen *et al*., 2001). The only exception was the new ET, a pedunculagin derivative, that was found to be the main ET in the leaves of white and mountain birches, being totally absent from the foliage of the other two species. The seasonal variations in the contents of individual ETs are shown to be much more complex than that of GGs. Possibly the only linear trend is the accumulation of the end product of the ET-pathway, i.e. 2,3-(*S*)-HHDP-glucose (Salminen *et al*., 2001). Accordingly, this end product accumulated seasonally in the leaves of white, mountain and dwarf birches ($P<0.05$), being present only in non-significant levels in the foliage of silver birch. As a remarkable indication of the conformity of ET synthesis in birch, the order of the main and minor ETs was

pretty much the same in all species despite the great differences in the levels of ET synthesis. In white and mountain birches, the newly found pedunculagin derivative was the main ET, otherwise the content of pedunculagin was followed by that of tellimagrandin I, presumably due to their central positions in the ET-pathway (Salminen *et al.*, 2001). *B. nana* was the only species where in addition to the temporally increased content of 2,3-(*S*)-HHDP-glucose, also the contents of other ETs like pedunculagin and casuarictin increased significantly (see Table IV). This highlighted the ET specificity of *B. nana* over the other birch species, in addition to it having the highest content of ETs in general.

Contents and seasonal variations of insoluble ellagitannins

The presence of insoluble ETs in all four birch species was confirmed for the first time by the appearance of ellagic acid after the MeOH/HCl hydrolysis of birch leaf insoluble residues (see Tables I-IV). Since any of the hydrolysis did not yield either gallic acid or methyl gallate even in trace amounts, the absence of insoluble GGs in birch leaves was evidenced as well. Furthermore, the identity of insoluble ETs can thus be limited to compounds like pedunculagin and 2,3-(*S*)-HHDP-glucose that do not have any galloyl groups and cannot therefore yield gallic acid, but only ellagic acid after hydrolysis. The content of insoluble ETs decreased in the foliage of *B. pendula* ($P<0.05$), but increased in *B. pubescens* ($P<0.01$) between the two collection dates. To confirm our assumption of increasing insolubility of ETs during the growing season at least in white birch, we analysed insoluble residues of the same white birches, of which data are shown in Table II, at ten additional points of the growing season. The results were calculated as amounts of insoluble ETs per one leaf (Fig. 1) to exclude the confounding effects of increasing leaf biomass, especially in young and rapidly growing leaves (Salminen *et al.,* 2001). Fig. 1 indicates a clear and increasing formation of insoluble ETs during the growing season and that the rate of insolubilisation coincided the leaf growth. Therefore insoluble ETs could be related to temporally increasing physical changes in birch leaves, such as e.g. leaf toughness that has been shown to be partly responsible for the herbivore defence of mature mountain birch foliage (Ossipov *et al.*, 2001). The same pattern of increasing amounts of insoluble ETs per one leaf between the two collection dates was obtained for mountain and dwarf birches as well (data not shown).

Ecological and biochemical implications of the contents of HTs in different birch species

High levels of total GGs in mountain birch foliage have been shown to reduce the physiological efficiency of digestion and the growth of the fourth instar larvae of *Epirrita autumnata* (Kause *et al.*, 1999). Similarly, the hydrolysis product of ETs, i.e. ellagic acid, has been shown to act as a growth inhibitor for the first instar larvae of the tobacco budworm *Heliothis virescens* (Klocke *et al.*, 1986). Therefore it could be generalised that the higher is the content of GGs and ETs in foliage, the higher is the ability of that plant to defend itself against various herbivores. In this sense, it is obvi-

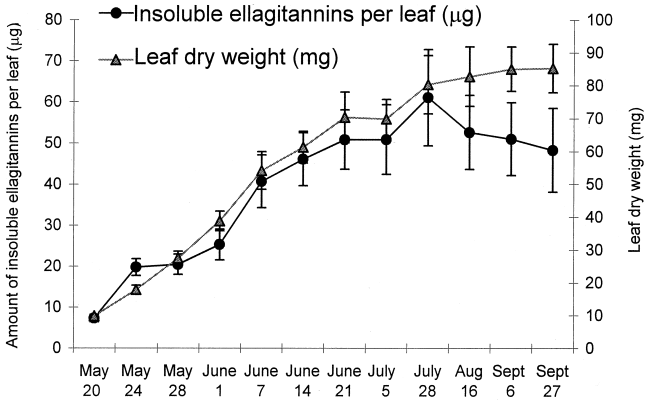

Fig. 1. The seasonal variations in birch (*Betula pubescens*) leaf dry weight and in the amount of insoluble ellagitannins per one leaf. Values are means of ten trees ± SE.

ous that silver birch is not relying its herbivore resistance on HTs that were barely detectable in its foliage. However, the level of an individual HT in the foliage is not necessarily linearly correlated with insect performance, and the rate of HT hydrolysis may differ greatly between the compounds (Salminen and Lempa, submitted). While considering ETs as sources of ellagic acid, it is noteworthy that the 4,6-HHDP-group is more easily cleaved out of ETs than the 2,3-HHDP (Salminen *et al.*, 2001). Therefore ellagic acid is formed easiest from compounds having the 4,6-HHDP group. This was confirmed by studying the hydrolysis of pedunculagin and the new pedunculagin derivative in detail. Both compounds resulted in 2,3-(*S*)-HHDP-glucose after hydrolysis in 100 °C water, but while pedunculagin gave ellagic acid already in water hydrolysis, the pedunculagin derivative did that only after more strong hydrolysis with MeOH/HCl. Therefore pedunculagin can be considered as the most important ET in white and mountain birches, although its level is somewhat lower than that of the pedunculagin derivative. Foliage of dwarf birch, on the other hand, contains clearly the highest amounts of pedunculagin, being thus an interesting object for future studies on plant-herbivore interactions.

While mountain birch is known to be a hybrid of white and dwarf birches (Vaarama and Valanne, 1973), also its HT content falls between the HT contents of these two "mother" species. Interestingly, as the new pedunculagin derivative can be found only in the foliage of white and mountain birches, but not in dwarf birch, the ability of mountain birch to synthesise this compound has to be of white birch origin. Since the distribution of the main HTs was otherwise alike in all species, it is most likely that the factors limiting the HT biosynthesis, e.g. in silver birch, were located in the very early steps of that pathway, i.e. in the formation of gallic acid (Ossipov *et al.*, submitted), the common precursor of HTs (Gross, 1999).

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