Seasonal variation in the content of hydrolysable tannins in leaves of *Betula pubescens* 

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Abstract

The contents of 13 hydrolysable tannins in the leaves of white birch (*Betula pubescens* L.) trees were analysed at twelve stages throughout the growing season. All individual galloylglucoses, from 1-O-galloyl-β-D-glucopyranose to 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose, accumulated in young leaves, while ellagitannins showed significantly variable seasonal trends. The major ellagitannin during the whole growing season was pedunculagin, while 2,3-(S)-HHDP-glucopyranose, the end product of the proposed ellagitannin pathway, accumulated in mature leaves. Relationships between the characteristics of seasonal variation in the contents of individual ellagitannins and their chemical structures were used to unravel the biogenesis of ellagitannins in birch leaves. Evidence of degradation of ellagitannins through hydrolysis during leaf growth and development is presented and implications for herbivory are discussed. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Tannins form a large group of polyphenolic plant constituents that differ from most other natural phenolic compounds in their ability to precipitate proteins from solutions (Niehaus and Gross, 1997). The protein precipitation capacity of tannins has been suggested as an important factor reducing the suitability of plants for herbivores (Feeny, 1970; Hagerman and Robbins, 1987; Appel and Schultz, 1994). However, along with other measures of biological activities of tannins, this capacity varies depending on the chemical structure of the compound (Feldman and Sahasrabudhe, 1999; Feldman et al., 1999; Kilkowski and Gross, 1999). Thus the total content of tannins as such cannot serve as a measure of the biological activity of a plant sample. This holds true especially if seasonal variations in the contents of individual active and non-active tannins display different patterns even when their pooled content remains the same.

The tannins of terrestrial plants can be divided into two major structural groups: hydrolysable tannins (HTs) and proanthocyanidins (condensed tannins). HTs are characterised by a central polyol moiety (most often β-D-glucose) which is esterified with gallic acid (gallocatechins, GTs) or hexahydroxydiphenic acid (ellagitannins, ETs). When the glucose core is esterified with five or fewer galloyl groups, the resulting compounds are defined as GT precursors or simple galloylglucoses (GGs), not GTs. The biogenesis of GGs has been shown to start with 1-O-galloyl-β-D-glucose as the first metabolite and to proceed by a series of consecutive and position-specific galloylation steps leading to 1,2,3,4,6-penta-O-galloyl-β-D-glucose (reviewed by Gross, 1999). In contrast to GGs, the biosynthetic pathway for the formation of ETs has not yet been determined, although it is generally assumed that all ETs are derived from 1,2,3,4,6-penta-O-galloyl-β-D-glucose by a stepwise, enzyme mediated oxidative coupling pathway (Helm et al., 1997).

Rapidly growing tree leaves display drastic seasonal changes in physical and biochemical characteristics, as has been shown in a number of studies (Feeny, 1970; Haukioja et al., 1978; Hatano et al., 1986; Baldwin et al., 1987; Makkar et al., 1991; Nurmi et al., 1996; Ossipov
et al., 1997; Kause et al., 1999). Most of these studies, however, measured the total contents of tannin groups, such as GTs or proanthocyanidins. Only in a few cases have individual HTs been quantified during the growing season (Hatano et al., 1986; Ossipov et al., 1997). Even then the numbers of individual HTs or sampling dates have been quite limited, thus preventing the determination, e.g. of ellagitannin pathways. Nevertheless, the high content of total GTs has been shown to reduce the suitability of birch leaves for larvae of the geometrid moth *Epirrita autumnata* (Kause et al., 1999; Ossipov et al., 2000).

Recently, using the sensitive and selective method of negative ion HPLC–ESI-MS, we have identified fourteen GGs and twenty ETs in birch (*Betula pubescens* L.) leaves (Salminen et al., 1999). In the present study the main HTs were isolated and purified, and the results of HPLC–ESI-MS identification were confirmed with \(^1\)H and \(^{13}\)C NMR spectroscopy. The contents of thirteen HTs were analysed 12 times during the growing season, using HPLC–ESI-MS with ion trace analysis. Relationships between the characteristics of seasonal variation in the contents of individual HTs and their chemical structures were used to unravel the biogenesis of ellagitannins in birch leaves. The results obtained will presumably contribute to an understanding of the great variability in the suitability of birch leaves for herbivores in different stages of leaf development (Kause et al., 1999).

2. Results

2.1. Identification of hydrolysable tannins

Birch leaf HTs have been earlier identified with chromatographic, UV spectral and mass spectrometric methods (Salminen et al., 1999). The structures of the main HTs were now confirmed by \(^1\)H and \(^{13}\)C NMR techniques as 1-O-galloyl-\(\beta\)-d-glucose (1) (Saijo et al., 1990), 1,6-di-O-galloyl-\(\beta\)-d-glucose (2) (Saijo et al., 1990), 1,2,6-tri-O-galloyl-\(\beta\)-d-glucose (3) (Haddock et al., 1982a), 1,2,3,6-tetra-O-galloyl-\(\beta\)-d-glucose (4) (Haddock et al., 1982a), 1,2,3,4,6-penta-O-galloyl-\(\beta\)-d-glucose (5) (Saijo et al., 1990; Haddock et al., 1982a), casuaricin (6) (Gupta et al., 1982), tellimagrandin I (7) (Wilkins and Bohm, 1976), pedunculagin (8) (Feldman and Smith, 1996; Lee et al., 1992) and 2,3-(S)‐HHDP-glucose (9) (Lee et al., 1992). Tellimagrandin II (10) and isostrictinin (11) were identified on the basis of UV and mass spectra (Salminen et al., 1999) and retention times (Hatano et al., 1986; Okuda et al., 1982).

It has been proposed that the metabolic pathway of ETs includes, in addition to 6, another galloyl-bis-HHDP-glucose isomer, namely potentillin (12) (Haddock et al., 1982b; Haslam, 1992). Haddock et al. (1982b) also reported the difficulty of separating 6 and 12 by chromatographic methods. We found birch leaves to contain two major isomers of galloyl-bis-HHDP-glucose that elute closely in HPLC (R,t 19.8 and 20.2). The earlier eluted isomer was identified as 6 by NMR (see above), and thus the later one must have been 12.

The identity of 1,2,3-tri-O-galloyl-4,6-(S)-HHDP-\(\alpha\)-d-glucose (13) was proposed on the basis of UV and mass spectra (Salminen et al., 1999) and of seasonal changes in its content relative to its \(\beta\)-1-galloylated isomer, 10. Likewise, the chirality of the HHDP moieties located at the 2,3- or 4,6-positions of the glucose core of natural ellagitannins has been reported to be invariably in the (S)-series (Feldman and Smith, 1996; Khanbabae and Lötzerich, 1998).

2.2. Seasonal trends in the content of hydrolysable tannins

Seasonal variations in the contents of birch leaf galloylglucoses are shown in Fig. 1. The maximum level was found in the youngest leaves. During leaf maturation the contents of all individual GGs per unit leaf weight decreased rapidly. However, their amounts per leaf increased, with a maximum between 28 May (1) and 7 June (5) (data not shown). This implies that the synthesis of GGs continued during the time when their content per unit leaf weight decreased due to dilution of GGs into the increasing leaf mass (Koricheva, 1999). Interestingly, after the short shoot leaves had reached their final size (after 5 July), clear increases were observed in the contents of all GGs except 1 (see Fig. 1).

In contrast to GGs, the contents of individual ETs showed variable seasonal trends (see Fig. 2a–d). This was especially obvious after excluding the rapid increase in leaf biomass during May and early June from the data by calculating the amounts of ETs per leaf (see Fig. 2e–h). For instance, while the contents of 6 and 8 seemed to remain at almost stable levels between 20 May and 7 June (Fig. 2b and c), their actual amounts per leaf increased significantly (Fig. 2f and g).

The order of individual ETs within the proposed ET pathway (Fig. 3) was determined from the relationships between the seasonal variation in the contents of individual ETs and their chemical structures. Plotting pairs of structurally or biogenetically related ETs showed that in the early stages of the growing season the balance was in favour of the predecessor, while during leaf maturation the balance switched towards the successor metabolite (for examples see Fig. 2).

Such ETs as 6, 10 and 12 disappeared totally from the leaves of four birch trees already at 6 September, while the main patterns of HT variation were the same in all experimental trees. There were, however, large differences in the levels of individual HTs between individual
trees, as can be seen from the relatively large standard deviation bars in Figs. 1 and 2.

3. Discussion

Our results show that the contents of individual birch leaf HTs follow distinct seasonal patterns. This variation in the contents of individual GGs was consistent with previous studies (Nurmi et al., 1996; Ossipov et al., 1997); the patterns of variation of individual ETs, on the other hand, were now determined for the first time. These results allow us to describe the biosynthetic pathways of the main GGs, and in particular ETs, in birch leaves.

1-O-Galloyl-β-d-glucose has been shown to be the first metabolite in the biogenesis of GGs, being formed from UDP-glucose and gallic acid (Gross, 1982). This same phenomenon was confirmed in our birch data: 1 was one of the major GGs in young leaves, representing more than 24% of the total content of GGs. Several studies have also suggested that 1 acts as a principal galloyl donor for a series of consecutive and position-specific galloylation steps leading to 5, the specificity of galloylations being usually 1-galloyl > 6-galloyl > 2-galloyl > 3-galloyl ≧ 4-galloyl (reviewed by Gross, 1999). The biosynthesis of the second metabolite, 2, requires two molecules of 1. The first molecule acts as a galloyl donor and the second as an acceptor. Thus the increase in the content of 2 would mean a clear decrease in the content of 1, especially if the rate of biosynthesis of 1 is simultaneously low. This was found to occur in mature leaves, after 5 July, as 2 became the major GG while 1 vanished almost totally (see Fig. 1b). The position-specificity of galloylations was proved to function in birch leaves as well; the other main GGs were identified as 3, 4 and 5.

In our study the final product of GG biosynthesis was 5, as indicated by the absence of hexagalloylglucoses even in trace amounts. The metabolism of 5 in birch leaves thus mainly relates to the biosynthesis of ETs and not to transformation into hexagalloylglucose or other higher galloylated GTs. This specificity of birch leaves could be the result of the lack of enzymes that catalyse the formation of GTs from 5 (Hofmann and Gross, 1990; Nienetz and Gross, 1998, 1999).

Denzel and Gross (1991) found that 5 inhibited the activity of sumac leaf galloyltransferase, which in turn activated the transfer of 1-galloyl from 2 to the 2-position of the same compound. A similar feedback inhibition may take place in the transfer of 1-galloyl from 1 as well. Accordingly, when the content of 5 declines in birch leaves, for example in the process of its active utilisation, this inhibition would diminish. This would be followed by a simultaneous decrease in the content of 1 and by an increase in the content of 2 and all the biosynthetically subsequent HTs, as was found in our study (see Figs. 1 and 2, after 5 July). The function of 2 as a galloyl donor in birch leaves is unlikely, since it would also have meant an increase in the content of 6-O-galloyl-β-d-glucose as the decacylated by-product; this was not observed (data not shown).

According to Yoshida et al. (1992) there is a direct position-specific dependence on the ease of cleavability of different galloyl as well as HHDP groups through hydrolysis: 1-galloyl > 2-galloyl > 3-galloyl ≧ 6- and 4-galloyl, 4,6-HHDP > 2,3-HHDP. The only galloyl group observed to be hydrolysed during this study was...
the 1-galloyl of ETs. This indicates that for instance in 7 the oxidative coupling between 2- and 3-galloyls to yield 8 is preferred compared to the hydrolysis of 2-galloyl. This hydrolysis would have yielded 3-O-galloyl-4,6-HHDP-glucose, which has an ungalloylated anomeric centre, thus producing two peaks in HPLC; this, however, was not observed. The more rapid cleavability of 4,6-HHDP compared to 2,3-coupled was confirmed, since 8 h hydrolysis of 8 in 100°C water yielded 9 and ellagic acid. It is interesting that while the 2,3-HHDP group of 8 is less prone to hydrolysis than the 4,6-HHDP, it is also more difficult to incorporate into the glucose core than the 4,6-HHDP in chemical syntheses of 8 (Feldman and Smith, 1996). The delayed degradation of 8, resulting in higher amounts of 9 in mature leaves only (see Fig. 2), could be due to the younger leaves having higher contents of certain GGs that inhibited the activity of ET degrading enzymes (Daniel et al., 1991).

In addition to hydrolysis, polymerisation and insolubilisation have also been suggested to decrease the content of ellagittannins (Sealbert et al., 1988; Peng et al., 1991; Klumpers et al., 1994; Viriot et al., 1994). According to Hatano et al. (1992), the intermolecular oxidative coupling producing oligomeric ETs in plants does not depend on a simple self-association of constituent monomeric tannins. Rather, it is controlled regiospecifically by enzymatic systems that vary among plant species. Presumably such enzymes were not present in our experimental trees, as polymerisation of
birch leaf ellagitannins, for instance to dimers, was not observed. The amount of insoluble ETs, on the other hand, has been shown to increase during birch leaf growth and development (Salminen et al., unpublished work). One explanation for the almost total disappearance of 6 and 12 in autumn leaves could have been the opening of their glucose ring and the formation of C-glycosidic ETs such as vescalagin and castalagin, the main ETs for instance of oak wood (Helm et al., 1997; Puech et al., 1999). However, such ring opening is not characteristic of birch leaf ETs as neither vescalagin or castalagin, nor their degradation products vescalin or castalin were detected in any stage of leaf development.

In the ecological literature, foliar tannins are traditionally viewed as precipitators of leaf proteins, which by this means increase the herbivore resistance of plants (Feeney, 1970). The formation of only monomeric ETs from 5, instead of highly galloylated GTs, suggests other functions for birch leaf hydrolysable tannins in addition to protein precipitation. Indeed, the accumulation of 9 in mature birch leaves is indirect proof of the existence and accumulation of ellagic acid, a proposed insect growth inhibitor (Klocke et al., 1986), in those same leaves as well. According to this view, HTs could play a dual role in plant-herbivore interactions, not only by a direct astringency-dependent deterrence of herbivorous insects, but also indirectly through their degradation products (Niehaus and Gross, 1997).

To sum up, we urge the need for studies in which individual HTs, including ETs, are considered as determinants of the changing suitabilities of birch leaves for herbivores (Kause et al., 1999). Such detailed studies will make it possible to determine whether these structurally diverse tannins, with possibly diverse biological activities, are truly effective defences against herbivores. In the words of Haslam (1988): “If tannins are so generalised in their action as supposed, it is very difficult to rationalise this generality with the very structural diversity found among the plethora of plant polyphenols — particularly those of the hydrolysable tannin class based on gallic acid and its derivatives.”

4. Experimental

4.1. Plant material

Short shoot leaves of 10 29- to 33-year-old white birches (Betula pubescens L.) of known seed origin were sampled in the Botanical Garden of the University of Turku (SW Finland) 12 times during the growing season of 1999. All short shoot leaves in a tree are
produced in the spring flush; thus the age of sampled leaves grew monotonously with the season. The first samples were collected when the leaves had just opened from the bud, while the last leaves sampled were senescent and about to fall. Since the major changes in the content of birch leaf HTs have been reported to take place in young leaves (Baldwin et al., 1987; Nurmi et al., 1996; Ossipov et al., 1997) the sampling interval varied from four days in the early spring to three weeks in the autumn. Samples of 10–15 leaves per tree were collected between 9 and 10 a.m. haphazardly from branches located 2–5 m above the ground. 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. Voucher specimens have been deposited at TUR under the following accession codes: TUR 360093 and TUR 360103.

4.2. Sample preparation

The freeze-dried birch leaves (300 mg dry wt) were extracted three times with 70% aq. Me$_2$CO. After removal of Me$_2$CO 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.

4.3. Isolation and purification of hydrolysable tannins

A large number of freeze-dried white birch leaves (1.8 kg dry wt) were extracted with 70% aq. Me$_2$CO (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 (fr 1–7), as previously described (Salminen et al., 1999). Individual HTs in the resulting fractions were purified further by chromatography on a Merck LiChroprep RP-18 column (44×3.7 cm i.d., 40–63 μm) using different gradients of CH$_3$CN in 1% aq. HCO$_2$H. This purification resulted in 1 (fr 1, 88 mg), 2 (fr 3, 48 mg), 3 (fr 5, 33 mg), 4 (fr 6, 39 mg), 5 (fr 7, 45 mg), 6 (frs 5 and 6, 23 mg), 7 (fr 5, 66 mg), 8 (fr 4, 35 mg) and 9 (fr 1, 19 mg).

4.4. Equipment and chromatographic conditions

4.4.1. 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). This instrument is equipped with an ion-spray (pneumatically assisted electrospray) interface and a Macintosh data system. The HPLC system consists of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT, USA) connected to a Series 200 autosampler. The eluate passes through a Merck Superspher 100 RP-18 column (75×4 mm i.d., 4 μm), and the UV trace is recorded with a 785 A 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).

4.4.2. $^1$H and $^{13}$C NMR

The $^1$H and $^{13}$C NMR spectra were recorded on a Jeol Lambda 400 MHz series spectrometer and a Bruker routine AM-200 NMR spectrometer, respectively. The spectra were run at ambient temperature in Me$_2$CO-d$_6$ (in some measurements a few drops of D$_2$O were added to enhance the solubility of the analyte). Chemical shifts were measured in relation to the solvent peak and converted to the TMS scale by adding 2.05 ppm ($^1$H NMR) and 29.92 ppm ($^{13}$C NMR).

4.5. Quantification of hydrolysable tannins

HTs of birch leaves were separated and quantified with HPLC–ESI-MS using ion trace analysis of deprotonated molecules ([M–H]$^-$) (Salminen et al., 1999). The mass selectivity of this method allows the quantitative analysis of HTs even though many of them are co-eluting in HPLC with other phenolic compounds present in birch leaf extracts. Calibration curves for quantification were prepared with known concentrations of 1, 2, 3, 4, 5 and 8. The contents of 6, 7, 9 and 12 were calculated as 8 equivalents; 10 and 13 as 5 equivalents; and 11 as 3 equivalent mg/g in dry wt basis. Differences in the ionisation of HTs between individual runs were standardised by monitoring the ionisation of chlorogenic acid as an internal standard.

The R$_s$ and values of deprotonated molecules used for the quantification of tannins were as follows: 9, 1.6 and 2.8 min (anomeric mixture), m/z 481; 1, 5.0 min, m/z 331; 8, 9.5 and 12.4 min (anomeric mixture), m/z 783; 7, 14.0 and 16.8 min (anomeric mixture), m/z 785; 2, 15.0 min, m/z 483; 11, 15.6 min, m/z 633; 3, 18.0 min, m/z 635; 6, 19.8 min, m/z 935; 12, 20.2 min, m/z 935; 10, 21.1 min, m/z 937; 4, 21.8 min, m/z 787; 13, 23.6 min, m/z 937; 5, 24.3 min, m/z 939.

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