Changes in Leaf Trichomes and Epicuticular Flavonoids during Leaf Development in Three Birch Taxa

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INTRODUCTION

Leaves of many plants are densely covered with glandular and non-glandular trichomes, which originate from epidermal cells (Werker, 2000). The indumentum of non-glandular trichomes and the lipophilic substances secreted by glandular trichomes (terpenes, lipids, waxes and flavonoid aglycones) serve as a barrier against various external factors, including herbivores and pathogens, UV-B radiation, extreme temperatures and excessive water loss (Ehleringer, 1982; Harborne, 1991; Wagner, 1991; Karabourniotis et al., 1993; Tattini et al., 2000; Werker, 2000). The development of leaf trichomes often begins at very early stages of leaf development, sometimes even before the leaf primordium can be distinguished. In some plants, the final number of trichomes is established during leaf differentiation (Ascensão and Pais, 1987), while in others new trichomes are formed throughout all the stages of leaf development (Maffei et al., 1989; Turner et al., 2000).

In many plant species trichome density is very high in young leaves but decreases rapidly with leaf expansion (Maffei et al., 1989; Werker et al., 1993; Pérez-Estrada et al., 2000). It has been suggested that, in young leaves lacking epidermis, trichomes and their exudates may serve as a functional analogue of the epidermis in mature leaves (Karabourniotis and Fasseas, 1996) since they play a similar protective role against biotic and abiotic factors such as water deficit (Ehleringer, 1982; Mauseth, 1988), insect herbivores (Levin, 1973; Juniper and Jeffree, 1983; Wagner, 1991), phytopathogenic fungi (Allen et al., 1991), and UV-B radiation (Day et al., 1993; Karabourniotis et al., 1993; Tattini et al., 2000). At later stages of leaf development, when the formation of the epidermis is completed, the functional role of the trichomes becomes less important, and they often senesce and shed. In some cases, however, trichomes remain viable and functional in mature leaves (Werker, 2000). Moreover, the composition of exudates produced by glandular trichomes may change with leaf age (Maffei et al., 1989; Werker et al., 1993).

The leaves and young shoots of birch (Betula) bear both glandular and non-glandular trichomes on their surface. The peltate resin glands on birch stem produce a mixture of phenolics and steroidal triterpenoids, which play an important role in birch resistance against mammalian herbivores (Reichardt et al., 1984; Rousi et al., 1991). It has
shown that the number of stem resin glands of *B. pendula* is

determined at the beginning of primary tissue growth, and

that the secretory activity of glands is higher on young


The secondary growth of later seasons separates the resin
droplets wider apart, and they gradually weather off. This

loss of primary chemical defense is compensated for by the

thickness and chemical constituents of the bark.

Unlike birch stem glands, birch leaf trichomes have so far

received considerably less attention, although recent studies

have suggested that they may play a role in birch resistance

against UV-B radiation (Kostina *et al.*, 2001), springtime

frost (Prozherina *et al.*, 2003) and insect herbivores (Rautio

*et al.*, 2002). A previous study of newly flushed leaves

in three birch taxa (*Betula pendula*, *B. pubescens* ssp.

*pubescens* and *B. pubescens* ssp. *czerepanovii*) revealed

significant differences in trichome structure and density among

taxa (Valkama *et al.*, 2003). It has also been demonstrated

that surface extracts of birch leaves contain flavonoid agly-

cones, and that composition and concentrations of these

compounds differ markedly among birch species (Valkama

*et al.*, 2003). However, changes in birch leaf trichomes and

flavonoid aglycones during birch leaf development have not

been studied before, although they may have important con-

sequences for birch adaptation to abiotic and biotic factors.

In the present paper, changes in trichome number and

ultrastructure, and in composition, concentrations and total

amount of flavonoid aglycones that occurred in leaves from

budburst to maturation, in *Betula pendula*, *B. pubescens* ssp.

*pubescens* and *B. pubescens* ssp. *czerepanovii*, are followed.

In particular, the following questions are addressed: (1) At

what stage of leaf development is the final number of tri-

chomes established? (2) Are birch trichomes present on leaf

surface during all stages of leaf development? (3) Do com-

position and concentrations of flavonoid aglycones change

throughout leaf development? (4) Are changes in trichome

density and concentrations of flavonoid aglycones due to

changes in leaf size or to changes in trichome number per

leaf and in synthesis of flavonoids?

**MATERIALS AND METHODS**

**Experimental trees**

The birch (*Betula L.*) trees used in this study represent

five different clones of *B. pendula*, five provenances

of *B. pubescens* ssp. *czerepanovii* and two seed origins

of *B. pubescens* ssp. *pubescens*. Details on tree origin are

provided in Valkama *et al.* (2003). The trees, growing in the

Botanical Garden of the University of Turku (60°26’N,

22°10’E), were aged 10 (*B. pendula*), 20 (*B. pubescens

ssp. *czerepanovii*) and 21 years (*B. pubescens* ssp.

*pubescens*) at the time of the study.

**Leaf samples**

Leaves from short shoots were collected at the following

stages of leaf development:

Stage 1: 1-cm-long leaves (7 May for *B. pendula*; 14 May

for *B. pubescens* ssp. *pubescens*; 15 May for *B. pubescens

ssp. *czerepanovii*)

Stage 2: 2-cm-long leaves (11 May for *B. pendula*;

17 May for *B. pubescens* ssp. *pubescens*; 20 May for

*B. pubescens* ssp. *czerepanovii*)

Stage 3: 3-cm-long leaves (20 May for *B. pendula*;

22 May for *B. pubescens* ssp. *pubescens*; 27 May for

*B. pubescens* ssp. *czerepanovii*)

Stage 4: 4-cm-long leaves (28 May for *B. pendula*;

29 May for *B. pubescens* ssp. *pubescens*; 5 June for

*B. pubescens* ssp. *czerepanovii*)

Stage 5: fully expanded leaves (7 June for *B. pendula*;

6 June for *B. pubescens* ssp. *pubescens*; 12 June for

*B. pubescens* ssp. *czerepanovii*)

Stage 6: mature leaves (3 July for *B. pendula*; 5 July for

*B. pubescens* ssp. *pubescens*; 10 July for *B. pubescens

ssp. *czerepanovii*).

**Density and total number of trichomes per leaf**

Three leaves from each of the three to five trees per each

cloned, provenance or seed origin were sampled at each stage

of leaf development. Samples were prepared as described by

Valkama *et al.* (2003) and trichome densities were counted

under a light microscope from three frames (1.3 x 1.3 mm²)

per leaf. The density of non-glandular trichomes (leaf hairs)

was studied only in *B. pubescens* ssp. *pubescens* because in

*B. pendula* leaf hairs are sparse and in *B. pubescens* ssp.

*czerepanovii* they have uneven distribution within leaf and

high variation within and between leaves, trees and pro-

venances. The total number of trichomes per leaf was calcu-

lated by multiplying the number of trichomes per mm² by the

leaf area.

**Microscopy**

One leaf was collected from short shoots of three trees per

each of clone, provenance or origin at stages 1, 3 and 5 of

leaf development. Samples for light, transmission and scan-

ning electron microscopy were fixed and studied as de-

scribed by Valkama *et al.* (2003). The presence of small

plastids, vacuoles, lipid droplets and osmiophilic material

was recorded from each sample to estimate seasonal changes

in trichome structure and the secretion of exudates.

**Extraction, analysis and identification of leaf surface

flavonoids**

Ten leaves from each clone, provenance or origin were

collected for each stage of leaf development and then frozen

and stored at −80 °C. Five leaves from each tree were used for

chemical analyses, while the remaining five leaves were dried

for 48 h at 70 °C for measurement of the percentage of leaf dry

weight (d. wt). Leaf surface flavonoids were extracted and

analysed as described by Valkama *et al.* (2003). The presence of small

plastids, vacuoles, lipid droplets and osmiophilic material

was recorded from each sample to estimate seasonal changes

in trichome structure and the secretion of exudates.

**Statistics**

The statistical analyses were carried out using SPSS

10.0 for Windows. The chi-square test followed by the
Mann–Whitney U test was used to compare ultrastructural parameters among stages of leaf development. For analysis of the density and the total number of trichomes, the General Linear Model (GLM) repeated-measures procedure was used with leaf side (upper or lower) and stage of leaf development as within-subject factors, and taxa (for glandular trichomes) or origin (for non-glandular trichomes) as between-subject factors. Clone-, provenance- or origin-specific means were used for this analysis.

Following the established practice in the ecological literature, the term ‘concentration’ is used to refer to the mass of a particular compound per unit of leaf mass (mg g⁻¹ d. wt; Koricheva, 1999), although the chemically correct term would be ‘content’. The mass of a compound per leaf (µg per leaf) is referred to as ‘amount’ (Riipi et al., 2002). The total amount of flavonoid aglycones per leaf was calculated as the total concentration of flavonoid aglycones multiplied by the mean dry mass of the leaf. The total concentration of flavonoid aglycones per mm² was calculated as the total amount of flavonoid aglycones per leaf divided by the mean leaf area. Pearson’s product-moment correlation coefficients were calculated between the average density of glandular trichomes on the upper and lower leaf sides and concentrations of individual compounds per mm² for each species. Clone-specific means for B. pendula (n = 5) or provenance/origin-specific means for both subspecies of B. pubescens (n = 7) were used for those correlations.

Changes in concentrations and total amount of surface flavonoids during leaf development were analysed separately in B. pubescens and B. pendula, since the composition of epicuticular flavonoids differs between the two species. The GLM repeated-measures procedure was used with stage of leaf development as the within-subject factor and subspecies (for B. pubescens) or clone (for B. pendula) as the between-subject factor. Changes in the total concentrations (mg g⁻¹ d. wt) and total amount of total flavonoid aglycones per leaf were examined by means of graphical vector analysis (Koricheva, 1999). This method makes it possible to distinguish whether shifts in concentrations are due to changes in compound synthesis or in biomass accumulation.

**RESULTS**

**Density and total number of glandular trichomes**

The density of glandular trichomes decreased throughout leaf development in all birch taxa examined, although to a different extent depending on stage of leaf development and taxon, as indicated by significant Stage × Taxa interaction (F₁₀,₃₀ = 8.5, P < 0.001; Fig. 1). For example, trichome density declined significantly in B. pendula from stage 1 to stage 3, whereas in B. pubescens ssp. pubescens it decreased significantly from stage 1 to stage 2. During later stages of leaf development, trichome density declined slightly and non-significantly in these taxa. In B. pubescens ssp. czerepanovii, it declined non-significantly for all stages of leaf development. Moreover, during leaf expansion, trichome density on the lower leaf side was higher in B. pubescens ssp. pubescens than in ssp. czerepanovii. However, when leaves reached their final size, there was no

![Fig. 1](image1.png)  
**Fig. 1.** Density of glandular (solid lines) and non-glandular trichomes (dotted lines, for B. pubescens ssp. pubescens only) on upper (A) and lower (B) sides of birch leaves during leaf development. Symbols represent clone-specific means for B. pendula (n = 5), provenance-specific means for B. pubescens ssp. czerepanovii (n = 5) or origin-specific means for B. pubescens ssp. pubescens (n = 2) ± s.e. Symbols marked by different letters are significantly different among stages of leaf development at P < 0.05.

![Fig. 2](image2.png)  
**Fig. 2.** Total number of glandular and non-glandular trichomes on leaves of different birch taxa. Bars represent clone-specific means for B. pendula (n = 5), provenance-specific means for B. pubescens ssp. czerepanovii (n = 5) or origin-specific means for B. pubescens ssp. pubescens (n = 2) ± s.e. Bars marked by different letters are significantly different among taxa within leaf side at P < 0.05. Bars marked by asterisks are significantly different among leaf sides within taxon at P < 0.05.
difference in glandular trichome density among birch taxa (Fig. 1).

Unlike trichome density, the total number of glandular trichomes per leaf did not change during leaf development in any of the birch taxa studied ($F_{5,30} = 2.3, P = 0.065$), although it varied significantly among taxa ($F_{2,6} = 15.7, P = 0.004$) and between leaf sides depending on taxon ($F_{2,6} = 13.3, P = 0.006$, Fig. 2). For example, on the upper leaf surface, the total number of glandular trichomes was higher in *B. pendula* than in both subspecies of *B. pubescens*. On the lower leaf surface, it was higher in *B. pendula* and in *B. pubescens* ssp. *pubescens* compared with that of ssp. *czerepanovii*. In addition, the total number of glandular trichomes was significantly higher on the lower leaf side than on the upper leaf side in *B. pendula* and *B. pubescens* ssp. *pubescens*, whereas the difference in their number was non-significant between leaf sides in ssp. *czerepanovii* (Fig. 2).

The density and total number of non-glandular trichomes were significantly higher ($P < 0.001$) on the upper leaf side than on the lower leaf side (Fig. 1, dotted line, and Fig. 2). During leaf development, the density of non-glandular
trichomes significantly decreased ($F_{5,25} = 14.32, P < 0.001$), but the total number of non-glandular trichomes did not change ($F_{5,25} = 1.32, P = 0.290$).

**Structural changes in glandular trichomes**

Light microscopy showed that glandular trichomes were already fully developed and differentiated into cortical and medullar cells in 1-cm-long birch leaves, which still lacked differentiated palisade and spongy parenchyma and epidermal cells (Fig. 3A). At this stage of leaf development, glandular trichomes had a spherical shape and intact cuticle (Fig. 3D). In 3-cm leaves, there was differentiation between palisade and spongy parenchyma, and the epidermis was already formed (Fig. 3B). In the cortical cells of glandular trichomes, central vacuole occupied almost the whole volume of the cytoplasm and the cuticle was slightly detached from the cells (Fig. 3B). When the leaf reached its final size, trichomes exhibit features of the post-secretory phase: flattened shape, degenerated cytoplasm and detached cuticle of some trichomes (Fig. 3C and E).

TEM observations revealed further developmental changes and among-taxon differences in glandular trichome ultrastructure. The proportion of cells with small plastids in *B. pendula* and *B. pubescens* ssp. *pubescens* was high in 1–3-cm leaves, but decreased drastically in fully expanded leaves (Fig. 4A). In *B. pubescens* ssp. *czerepanovii*, glandular cells with small plastids were observed only in 1-cm leaves, but not in older leaves (Fig. 4A). The proportion of glandular cells with small vacuoles declined during leaf

**Fig. 3.** Light (A–C) and SEM (D and E) micrographs of glandular trichomes of *B. pendula* at different stages of leaf development. (A and D) 1-cm leaves. Young glandular trichomes have a spherical shape, their cells are differentiated into cortical and medullar cells and are covered by intact cuticle. No differentiation between palisade and spongy parenchyma could be distinguished, and epidermis is not completely developed. (B) 3-cm leaf. Cuticle is slightly detached from cortical cells. Vacuole occupied almost the whole volume of the cytoplasm. Leaf mesophyll is differentiated into palisade and spongy parenchyma, epidermis is already formed. (C and E) Fully expanded leaves. Aged trichomes are flattened, covered with detached cuticle (arrows). Scale bars: A, B and C = 50 μm; D and E = 10 μm. CC, cortical cells; MC, medullar cells; PP, palisade parenchyma; SP, spongy parenchyma; E, epidermis; V, vacuole.
development in all birch taxa (Fig. 4B). The proportion of glandular cells with osmiophilic material decreased significantly in B. pubescens ssp. czerepanovii and tended to decline in ssp. pubescens, whereas, in B. pendula, trichome cells lacked osmiophilic material during all stages of leaf development (Fig. 4C). In contrast, the proportion of cells with lipid droplets increased with leaf age in B. pubescens ssp. czerepanovii, while no changes were observed in B. pendula and B. pubescens ssp. pubescens (Fig. 4D).

Epicuticular flavonoids

The composition of flavonoid aglycones did not change during leaf development. The same 12 and 6 compounds, which were present in newly flushed leaves of B. pubescens and B. pendula, respectively (Valkama et al., 2003), occurred on the birch surface regardless of the leaf developmental stage. In contrast, concentrations (mg g⁻¹ d.wt and mg mm⁻²) of flavonoid aglycones decreased during leaf development in all taxa examined (Fig. 5). Two compounds, flavonol methyl ether 2 and flavanone methyl ether, occurred at highest concentrations in both subspecies of B. pubescens (Fig. 5A and B). In ssp. czerepanovii, concentrations of these compounds decreased considerably during the first three stages of leaf development, but only slightly during later stages (Fig. 5B), whereas, in ssp. pubescens, concentrations of these compounds decreased moderately until stage 3, but markedly during later stages (Fig. 5A). In B. pendula, concentrations of tetrahydroxyflavone dimethyl ether decreased five-fold from stage 1 (1-cm leaves) to stage 6 (mature leaves), while the concentrations of other compounds changed only slightly (Fig. 5C).

Changes in total concentrations (mg g⁻¹ d.wt) and in total amount (µg per leaf) of flavonoid aglycones varied depending on the taxon, as indicated by significant Leaf stage × Taxa interaction (F_10,30 = 48.6, P < 0.001 and F_10,30 = 8.04, P < 0.001, respectively). In B. pubescens ssp. pubescens, the decrease in total concentrations of flavonoid aglycones in expanding leaves was accompanied by an initial increase in the total amount of flavonoids (Fig. 6A). The total amount was stabilized in stage 3 (22 May) and stage 4 (29 May) and then significantly decreased in mature leaves (5 July; Fig. 6A). In B. pubescens ssp. czerepanovii, total concentrations of flavonoid aglycones decreased with leaf development from 29.2 ± 3.9 mg g⁻¹ d.wt in 1-cm leaves to 3.2 ± 0.8 mg g⁻¹ d.wt in mature leaves (P < 0.05), but the total amount of flavonoids did not change (295 ± 51 µg per leaf in 1-cm leaves and 248 ± 54 µg per leaf in mature leaves; P > 0.05). Finally, in B. pendula the decrease in total concentrations of flavonoid aglycones was accompanied by a gradual increase in their total amount until leaves reached their full size (7 June) and then by non-significant decline in mature leaves (3 July; Fig. 6B).

Correlations between glandular trichome density and epicuticular flavonoids

In both subspecies of B. pubescens, glandular trichome density and concentrations of all 12 individual...
compounds per mm$^2$ were highly correlated through all stage of leaf development ($r = 0.77–0.99$, $P < 0.001$, $n = 7$). In $B. pendula$, concentrations of all of the compounds except apigenin and pentahydroxyflavone trimethyl ether were correlated with glandular trichome density through all stages of leaf development ($r = 0.62–0.92$, $P < 0.01$, $n = 5$).

**DISCUSSION**

The results presented here demonstrate that leaf trichomes in all birch taxa are differentiated at a very early stage of leaf development, prior to the differentiation of epidermis and mesophyll cells. During leaf development, the density of trichomes decreased in all Betula taxa studied. This decline was due to growth dilution in expanding leaves, since the total number of trichomes per leaf remained constant and neither the formation of new trichomes nor trichome shedding at later stages of growth occurred. Similarly, the number of resin glands on shoots of $B. pendula$ is determined at the beginning of primary tissue growth, and the formation of additional new glands at later stages of growth is highly unlikely (Lapinjoki et al., 1991).

Similarly to trichome density, total concentrations (mg g$^{-1}$ d. wt and mg mm$^{-2}$) of surface flavonoid aglycones decreased considerably during leaf development. In $B. pubescens$ ssp. $czerepanovii$ and $B. pendula$, this decrease was largely due to growth dilution in expanding leaves for all stages of leaf development, since the amount of
flavonoid aglycones (µg per leaf) either did not change (in B. pubescens ssp. czerepanovii) or increased with leaf age (in B. pendula). However, in B. pubescens ssp. pubescens, growth dilution occurred only during the first three stages of leaf development, whereas in mature leaves the decrease in the total concentration of flavonoid aglycones was observed together with the decline in their total amount per leaf. This might be due to reduction in their synthesis together with simultaneous degradation of the compounds or transformation into insoluble, cell-wall-bound forms. This indicates that flavonoid synthesis is at its highest level in young versus older leaves in all taxa studied, whereas its duration may vary among birch taxa. Moreover, glandular trichome density is strongly correlated with concentrations of most of the individual flavonoid aglycones (per mm²) through leaf development, suggesting the participation of glandular trichomes in production of surface flavonoids. The localization of flavonoid aglycones within glandular trichome cells was frequently demonstrated for other plant species (for references, see Wollenweber, 1984).

Furthermore, ultrastructural features of birch glandular trichomes change throughout leaf development. Cells of young birch glandular trichomes are characterized by many traits indicating high metabolic activity; the presence
of numerous small plastids, small vacuoles and osmiophilic material, which is likely to represent phenolics (Valkama et al., 2003). All the above features declined rapidly with leaf age. Therefore, it seems likely that the secretory phase of birch trichomes is relatively short (about 1–2 weeks) and coincides with leaf expansion. The release of the secreted material in young leaves probably occurs by the passage of exudates droplets through the intact cuticle, since no cuticular rupture was observed in young trichomes. A similar mode of secretion of exudates, which contains flavonoid aglycones, was suggested for Salvia blepharophylla (Bisio et al., 1999).

By the time the birch leaf reaches its full size and the formation of epidermis is completed, the glandular trichomes exhibit features of the post-secretory phase (flattened shape, degenerated cytoplasm and detached cuticle). At this phase, trichomes function mainly as storage organs allowing release of exudates on leaf surface by means of cuticular rupture, since high amounts of lipid droplets have been observed in their cells and total amount of surface flavonoids was the highest in fully expanded leaves of B. pendula.

Ultrastructural observations indicated that other types of chemical compounds were produced in glandular trichomes in addition to flavonoid aglycones. Osmiophilic material, which is likely to represent phenolics containing o-dihydroxy groups (Nielson and Griffith, 1978), was not secreted with exudates, but stored inside glandular trichome cells in both subspecies of B. pubescens (Valkama et al., 2003). During leaf development, the amount of osmiophilic material declined, nevertheless, 20–40% of cells in aged trichomes possessed it. Similarly, the presence of phenolics within hair cells has been reported for Olea leaves (Karabourniotis et al., 1992). It is possible that phenolics, occurring in glandular trichomes of B. pubescens, may be as important in birch resistance against herbivores as foliar phenolics (Kause et al., 1999; Haukioja et al., 2002).

To summarize, the final number of birch trichomes is already established in newly flushed leaves and does not change during leaf development. In contrast, the density of trichomes and concentrations of epicuticular flavonoid aglycones exhibit a rapid decline with leaf age. These results support the suggestion by Karabourniotis and Fasseas (1996) that the role of leaf trichomes is particularly significant during the early stage of leaf development when leaves still lack differentiated epidermis. At later stages of leaf development, glandular trichomes cease to synthesize exudates and function mainly as storage organs allowing release of exudates.

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LITERATURE CITED


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