Research Article

Degradation of chlorophyll and synthesis of flavonols during autumn senescence—the story told by individual leaves

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Abstract.   Autumn senescence of deciduous trees is characterized by chlorophyll degradation and flavonoid synthesis. In the present study, chlorophyll and flavonol contents were measured every morning and evening during the whole autumn with a non-destructive method from individual leaves of *Sorbus aucuparia*, *Acer platanoides*, *Betula pendula* and *Prunus padus*. In most of the studied trees, the chlorophyll content of each individual leaf remained constant until a phase of rapid degradation commenced. The fast phase lasted only ~1 week and ended with abscission. In *S. aucuparia*, contrary to the other species, the chlorophyll content of leaflets slowly but steadily decreased during the whole autumn, but rapid chlorophyll degradation commenced only prior to leaflet abscission also in this species. An increase in flavonols commonly accompanied the rapid degradation of chlorophyll. The results may suggest that each individual tree leaf retains its photosynthetic activity, reflected by a high chlorophyll content, until a rapid phase of chlorophyll degradation and flavonoid synthesis begins. Therefore, in studies of autumn senescence, leaves whose chlorophyll content is decreasing and leaves with summertime chlorophyll content (i.e. the leaves that have not yet started to degrade chlorophyll) should be treated separately.

Keywords: Bird cherry; chlorophyll breakdown; circadian rhythm; diurnal; maple; rowan; silver birch.

Introduction

During autumn senescence, deciduous trees shed their leaves, remobilizing nutrients, mainly nitrogen and phosphorus (for review, see Brant and Chen 2015), for winter storage. Senescence is a genetically controlled, energy-consuming process that ultimately leads to death of specific plant organs (for review, see e.g. Hopkins *et al.* 2007).

The course of autumn senescence in *Populus tremula* has been described by Keskitalo *et al.* (2005). The first visible sign is chlorophyll (Chl) degradation, followed by degradation of other macromolecules (e.g. carotenoids and proteins), nutrient remobilization and cessation of starch accumulation. Photosynthesis declines while functional chloroplasts turn into gerontoplasts, and mitochondria become the main energy producers

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(e.g. Collier and Thibodeau 1995; Andersson *et al.* 2004). However, a small number of chloroplasts remain intact and some photosystem II (PSII) centres stay functional until almost the end of senescence (Keskitalo *et al.* 2005; Moy *et al.* 2015). Ultimately, metabolic activity ceases, cytoplasm is degraded, the abscission zone forms and the leaf falls off.

In *P. tremula*, autumn senescence seems to be triggered only by the shortening of the photoperiod (Fracheboud *et al.* 2009), but in other species both the onset and duration of senescence are affected by additional factors, such as temperature (Fu *et al.* 2014; Gill *et al.* 2015). During the growth period, senescence of a whole plant or a plant organ can be triggered by different stresses, darkness or developmental stage. The cellular processes at the early phases of senescence have been shown to differ remarkably depending on the triggering factor but at later stages the regulation seems to be similar (for review, see e.g. Fischer 2012; Guo and Gan 2012; Springer *et al.* 2015). During autumn senescence, expression levels of many genes change (Bhalerao *et al.* 2003; Andersson *et al.* 2004; Wen *et al.* 2015). Generally, genes related to photosynthesis are down-regulated, but many stress-related genes, as well as genes involved in degradation processes or in mitochondrial functions, are up-regulated or stay constant.

Up to 75 % of leaf nitrogen is in chloroplasts (Peoples and Dalling 1988), both in thylakoid proteins and in stromal proteins like rubisco. A significant part of the nitrogen is found in chlorophyll-binding light-harvesting complexes (LHC), and degradation of chlorophyll prevents the production of singlet oxygen, a reactive oxygen species (ROS), by free chlorophylls released when the protein is degraded. Breakdown of chlorophyll begins with reduction of chlorophyll *b* to chlorophyll *a* and proceeds via multiple steps to a colourless linear tetrapyrrole (for review, see Hörtensteiner and Kräutler 2011). Fluorescent chlorophyll catabolites are able to produce singlet oxygen (e.g. Pružinská *et al.* 2007) and are moved to the vacuole where they lose their colour in an acid-catalyzed reaction (Hörtensteiner and Kräutler 2011). Derivatives of the non-fluorescent degradation products and side products of the degradation have been speculated to play roles as antioxidants and signal molecules (Christ and Hörtensteiner 2014).

The spectacular autumn colours are partly caused by exposure of carotenoids due to faster degradation of chlorophyll. In addition, some species also synthesize specific flavonoids, e.g. anthocyanins (for review, see Falcone Ferreyra *et al.* 2012) which are largely responsible for the red colours in senescing leaves. Anthocyanins are suggested to act as antioxidants or metal ion chelators, in blocking ultraviolet (UV) or visible radiation, or playing roles in defence and signalling (for review, see Archetti 2009a; Landi *et al.* 2015). The amounts of other flavonoid compounds, flavonols or flavonol glycosides, have been shown to increase during age-related senescence (Torras-Claveria *et al.* 2012) but their roles are poorly understood. In contrast to anthocyanins, flavonols do not absorb visible light. They are found in the cuticle and sometimes inside the cells and they block UV radiation (for review, see Solovchenko and Merzlyak 2008) and may act as ROS-scavengers or signal molecules (Pollastri and Tattini 2011). Flavonol glycosides have also been suggested to be involved in super-cooling of xylem parenchyma cells of *Cercidiphyllum japonicum* (Kasuga *et al.* 2008). The diversity of plant flavonoids may suggest that different compounds have different roles.

Even though autumn senescence is considered to be coordinated and synchronized at the organism level, neither all leaves of a tree nor all cells of a single leaf senesce simultaneously (e.g. Keskitalo *et al.* 2005). However, in many studies, autumn senescence is treated as an averaged phenomenon, and investigations that connect senescence of an individual leaf and the whole tree are rare. In the present study, we followed a number of single leaves of four trees, each representing a different species, *Sorbus aucuparia*, *Acer platanoides*, *Betula pendula* and *Prunus padus*, from late growth season through the whole autumn. The chosen trees are native to Northern Europe and all of them except *B. pendula* turn red during senescence (Archetti 2009b), probably due to the accumulation of anthocyanins. The aims of the study were to find out how the chlorophyll content of an individual leaf behaves during autumn senescence both on a day-to-day basis and during the circadian cycle, and to study the role of flavonols in tree senescence.

Methods

Plant material

Four deciduous trees, rowan (*S. aucuparia*; ~5 m tall), Norway maple (*A. platanoides*; ~10 m), silver birch (*B. pendula*; ~15 m) and bird cherry (*P. padus*; ~1 m), growing in a small park (Turku, Finland, 60°N, 22°E), were chosen for the measurements, and individual full-grown leaves from each tree were marked before the measurements started. The park is located on the top of a small hill, and the environment is sunny and dry. The park was not directly illuminated by street lights. Before the measurements started, several leaves from each tree (at 0.5–2 m height) were marked. *Acer platanoides* and *B. pendula* trees were not shaded by other trees much; though the lower leaves of *A. platanoides* marked in the present study were shaded by the upper leaves of the same tree. *Sorbus aucuparia* and *P. padus* trees were partially shaded by the other trees in the area, depending on the time of day and the date.

Weather data **[see Supporting Information—Fig. S1]** were obtained from Finnish Meteorological Institute; the weather station is located 7 km from the studied trees.

Pigment measurements

Optical measurements. Chlorophyll and epidermal flavonol contents were measured simultaneously *in vivo* using a non-destructive measurement device (Dualex Scientific™, Force-A, Paris, France). The indexes of chlorophyll and flavonols are defined as (http://www.force-a.com/en, 11 November 2016; for the exact formula for chlorophylls, see Cerovic *et al.* 2012):

$$Chlorophyll = \frac{transmitted\ near\ infrared\ light - transmitted\ red\ light}{transmitted\ red\ light}$$

$$Flavonols = Log\frac{near\ infrared\ fluorescence\ excited\ by\ red\ light}{near\ infrared\ fluorescence\ excited\ by\ UV-A}$$

The measurements were taken from four leaves consisting of 57 leaflets (1 measurement per leaflet) of *S. aucuparia*, 15 leaves (3 measurements per leaf) of *A. platanoides*, 30 leaves belonging to six branches (2 measurements per leaf) of *B. pendula* and 11 leaves (6 measurements per leaf) of *P. padus*. The measurements were conducted daily (excluding weekends) at sunrise and at sunset, from 24 August 2015 to 22 October 2015 or until the leaf or leaflet fell off or was damaged by an external factor like herbivory.

Isolation of pigments. The chlorophyll contents of *S. aucuparia* leaflets were measured also with a destructive method during 23 August–25 September 2009. Ten leaf discs were collected twice a day, at sunrise and at sunset, in a predetermined random order from 125 previously marked leaves. Chlorophylls were extracted by incubating the leaf discs in *N,N*-dimethylformamide (DMF) for 2–3 days in darkness at 4 °C, after which the amount of chlorophyll was quantified spectrophotometrically according to Porra *et al.* (1989). In the measurements done in 2015, chlorophylls of several leaves from all four trees with varying greenness were measured both with Dualex and extracted in DMF to validate and calibrate the non-destructive method for senescent leaves.

High-performance liquid chromatography analysis of phenolic compounds

Leaves were dried at room temperature for 24 h in a desiccator and stored at 4 °C. Large veins were removed and the rest of the leaf material (dry weight 5–50 mg) was ground to small pieces, 1 mL of methanol was added, the sample was vortexed and incubated over night at 4 °C in darkness. Thereafter, the sample was vortexed again, centrifuged, and the supernatant was removed. Another 1 mL of methanol was added, the sample was vortexed and centrifuged and the supernatant was combined with the previous extract. The sample was filtered through a 0.2-μm polytetrafluoroethylene filter and assayed with high-performance liquid chromatography (HPLC; Agilent 1100 Series, Agilent Technologies, Germany) according to Seal (2016), except that a LiChroCART column (RP-18, 5 μm particle size; 125 × 4 mm) was used, and the gradient elution time was increased from 55 to 70 min to remove carotenoids from the column. Estimates of the total amounts of flavonols and other phenolic compounds were calculated by summing the absorbance at 355 nm of each peak **(see Supporting Information—Fig. S2** for the retention times and the spectra), and normalized by dividing by the dry weight of the sample. Due to different grinding methods, the amount of phenolics cannot be compared between the species. The compounds were manually classified as a flavonol, flavanone, other flavonoid, phenolic acid or other related compound according to their retention times and absorption spectra **[see Supporting Information—Fig. S2]**.

Statistical analyses

Mann–Whitney *U* test and Student's *t*-test were performed in Excel (Microsoft). Two-tailed distributions were used, and significant differences are reported only when the null hypothesis was rejected with probability of 99 % or higher. The sample sizes are given in respective figures. Analysis of variance (ANOVA) was calculated with R software (R Core Team 2014).

For the analysis of the circadian variation in the change of chlorophyll and flavonoid contents of the leaves, each measurement from all four trees was manually classified to separate samples in which rapid chlorophyll degradation had started or to samples in which rapid chlorophyll degradation had not started (stable group). In *S. aucuparia*, leaves 1 and 2, three leaflets of leaf 3 and two leaflets of leaf 4 were classified to the rapid group from the beginning of the analysis. All other leaflets of leaf 3 belonged to the rapid group from 15 September on, and all other leaflets of leaf 4 belonged to the rapid group from 21 September on. In *A. platanoides*, all leaves belonged to the rapid group from 12 September on. In *B. pendula*, two leaves of the first leaf group were classified as rapid from 28 August on, two leaves from 25 September on and two from 16 October on. In *P. padus*, all leaves of the second analysis

group were classified as rapid. All other measurements belonged to the stable group.

Results

Chlorophyll contents of individual leaves of senescing trees remained stable until onset of rapid degradation

Chlorophyll and flavonol contents of individual leaves of *S. aucuparia*, *A. platanoides*, *B. pendula* and *P. padus* were recorded with a non-destructive method twice a day from 23 August 2015 to 23 October 2015 (Figs 1–4). The autumn weather was typical for the region, with day temperatures falling from around 15 to 10 °C during the experiment, and the first night frosts in mid-October [see **Supporting Information—Fig. S1**].

The optical method was validated for senescing leaves and was found to work well (see Appendix 1). In addition, the chlorophylls of leaflets of *S. aucuparia* were analysed with a destructive method during autumn 2009 [see **Supporting Information—Fig. S3**]. The chlorophyll content decreased in all four species during the autumn but in different manners (Figs 1–4). The chlorophyll contents of *S. aucuparia* leaflets slowly decreased during the whole measurement time, but 3–5 days prior to abscission, the rate of degradation increased (Fig. 1A). Analysis of the earlier destructive measurements reveals similar behaviour,

as the chlorophyll contents of most *S. aucuparia* leaflets decreased slowly while a few leaflets had a low chlorophyll content [see **Supporting Information—Fig. S3**].

In contrast to *S. aucuparia*, virtually no decrease in chlorophyll content occurred in any individual leaves of the other measured trees before the onset of fast chlorophyll degradation that led to the abscission of the leaf (Figs 2–4). In *A. platanoides* (Fig. 2A) and *P. padus* (Fig. 4A), the fast degradation of chlorophyll started in a concerted manner in all studied leaves (on 13 October 2015). *Acer platanoides* leaves were detached 8–10 days and *B. pendula* leaves 6 days after the onset of rapid chlorophyll degradation (Figs 2A and 3A). The data for *P. padus* are not conclusive but suggest similar timing as in *A. platanoides* (Fig. 4A).

In the leaves of *P. padus* and *B. pendula*, chlorophyll degradation begins in seemingly random order [see **Supporting Information—Fig. S4**]. In the data measured during the autumn, chlorophyll degradation started at different times in different *B. pendula* leaves (Fig. 3A), and the leaves belonging to same branches did not behave similarly. On the contrary, in *S. aucuparia*, the timing of chlorophyll degradation differed between leaves but leaflets belonging to the same leaf had a more uniform timing (Fig. 1A). Lower leaves lost chlorophyll before the upper leaves (Fig. 1A). According to our observations, early signs of senescence in lower leaves is a feature shared by some but not all *S. aucuparia*
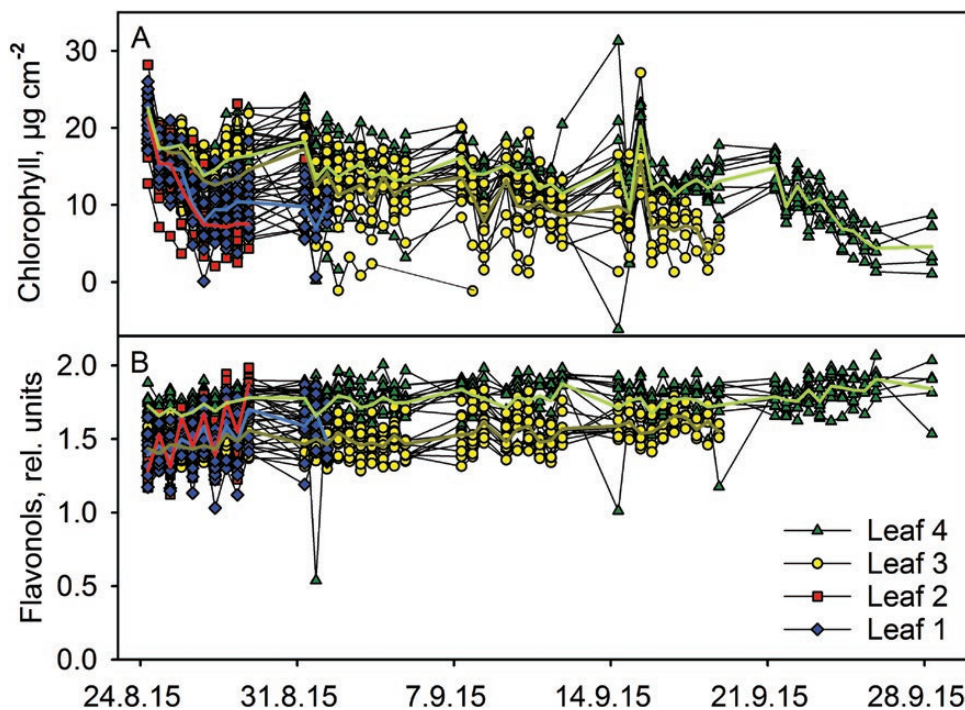

Figure 1. Chlorophyll (A) and flavonol (B) contents during autumn senescence in 57 *Sorbus aucuparia* leaflets belonging to four leaves. The numbering of the leaves indicates their respective order in the tree, the smallest being lowest. The symbols with the same colour in (A) and (B) represent the same leaf, and each line shows an average of the leaflets of one leaf.

trees [see **Supporting Information—Fig. S4**]. *Acer platanoides* leaves used in the present study were from the lowest branches and in *A. platanoides* trees the upper, more exposed leaves are usually the first ones to turn red [see **Supporting Information—Fig. S4**].

Autumnal changes in flavonol content depend on species and time

The flavonol contents of *S. aucuparia* leaflets slightly increased during the whole autumn and the increase seemed to speed up with decrease in the chlorophyll content (Fig. 1B). Increase in flavonols coincided with chlorophyll degradation also in *A. platanoides* and *B. pendula* (Figs 2B and 3B). In *A. platanoides* leaves, a slow constant increase in flavonol content continued for the whole autumn but the rate clearly increased a few days before chlorophyll degradation started (Fig. 2B). In *B. pendula* leaves, there was little or no increase in flavonols as long as the chlorophyll content of the leaf remained stable, but the flavonol content increased very rapidly when chlorophyll was degraded. This behaviour was especially clear during mid-autumn (Fig. 3B). In contrast to the other studied trees, the flavonol content of *P. padus* leaves did not change during the autumn (Fig. 4B). The same trend was observed when the rates of change in flavonol and chlorophyll contents in 2.5 days (calculated as a slope of a line) were plotted against each other [see **Supporting Information—Fig. S5**].

Diurnal changes in chlorophyll and flavonol contents

To analyse diurnal changes in chlorophyll or flavonol contents, we calculated how much the amounts of these compounds changed during the nights or the days (by subtracting the evening value from the value of the next morning and the morning value from the value measured next evening; Fig. 5). The analysis was done for all the individual leaves or leaflets of the four trees, separately for those measurements from leaves or leaflets with stable chlorophyll content and for measurements from leaves with decreasing chlorophyll content.

On the average, the chlorophyll contents of *S. aucuparia* leaflets with fairly stable chlorophyll levels did not change during the nights (Fig. 5A), whereas the change in the chlorophyll content during the days was negative, indicating that the rate of chlorophyll degradation exceeded the rate of synthesis (Fig. 5A). The difference between day and night was statistically significant, suggesting that in *S. aucuparia*, net degradation of chlorophyll occurs only during daytime. The daytime degradation led to the net loss of chlorophyll as, in contrast to the other species, the chlorophyll content of *S. aucuparia* leaves slowly decreased for the whole autumn (Fig. 1 and **Supporting Information—Fig. S3**). Also in *B. pendula* leaves with stable chlorophyll content, the average changes in chlorophyll levels during the nights and the days were significantly
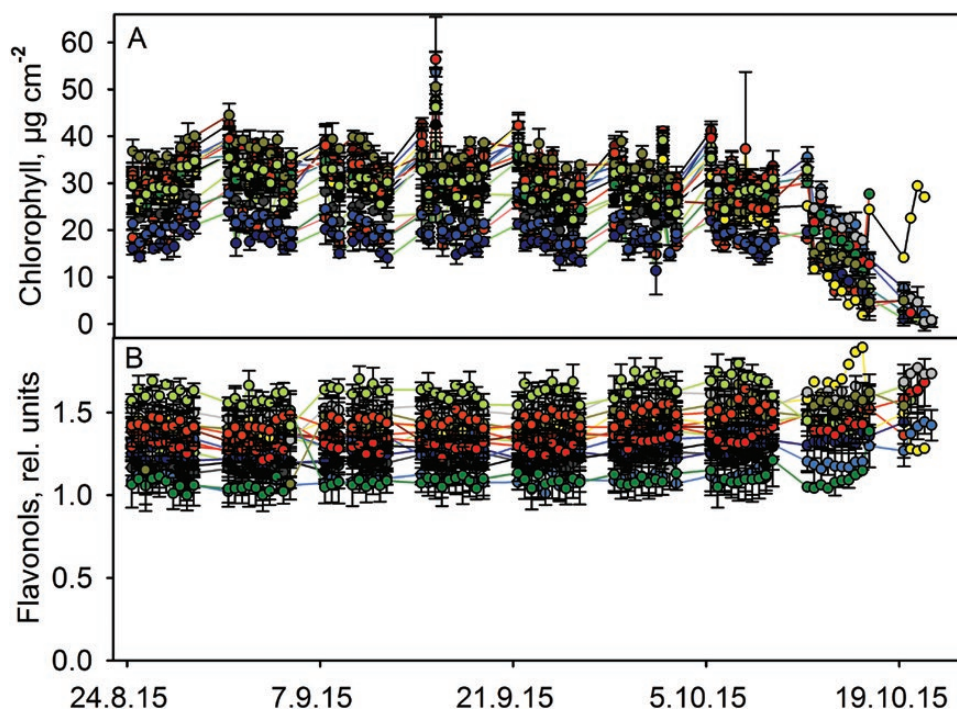

Figure 2. Chlorophyll (A) and flavonol (B) contents during autumn senescence in 15 *Acer platanoides* leaves, shown with different colours. The curves with the same colour in (A) and (B) represent the same leaf. Error bars show SD calculated from three measurements from the same leaf.
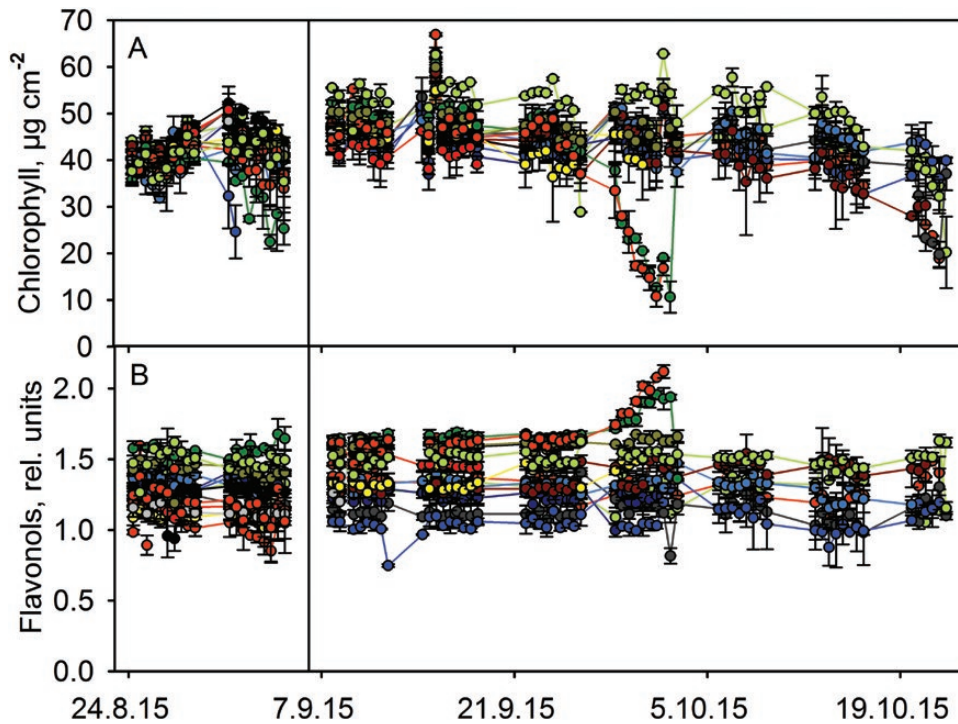
Figure 3. Chlorophyll (A) and flavonol (B) contents during autumn senescence in 15 (first set) and 14 (second set) *Betula pendula* leaves, shown with different colours. Measurements before and after the vertical line are from different leaves; the first set of leaves was abandoned because of extensive damage by insect herbivores. The curves with the same colour in (A) and (B) represent the same leaf. Error bars show SD calculated from two measurements from the same leaf.

Figure 4. Chlorophyll (A) and flavonol (B) contents during autumn senescence in eight (first set) and three (second set) *Prunus padus* leaves, shown with different colours. Measurements before and after the vertical line are from different leaves; the first set of leaves was abandoned because of massive damage to the leaf marks by an herbivore or a passer-by. The curves with the same colour in (A) and (B) represent the same leaf. The error bars show SD calculated from six measurements from the same leaf.
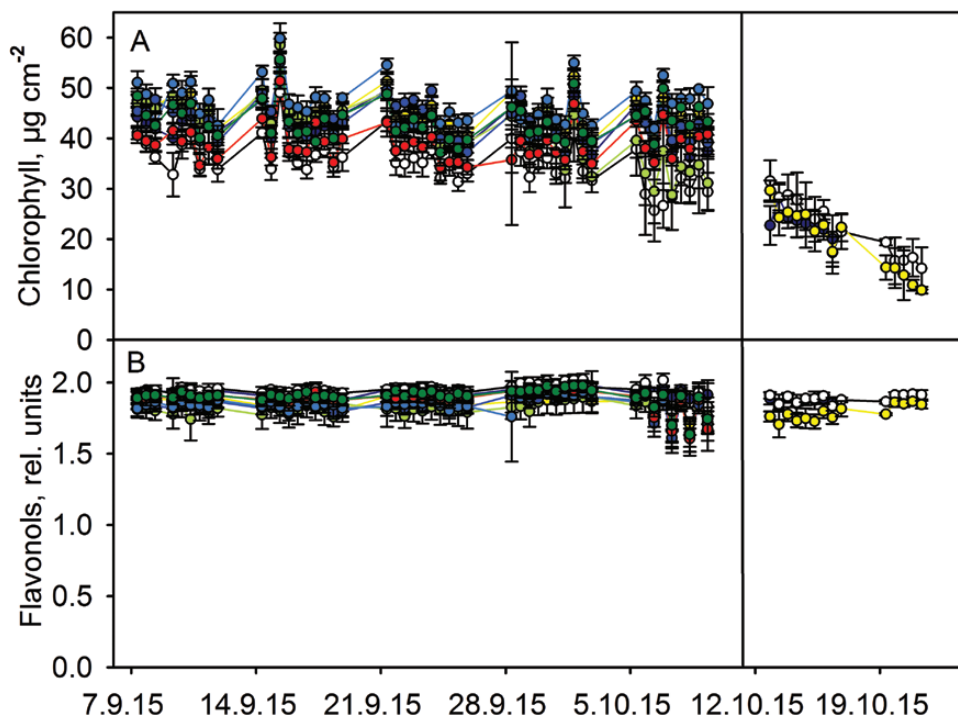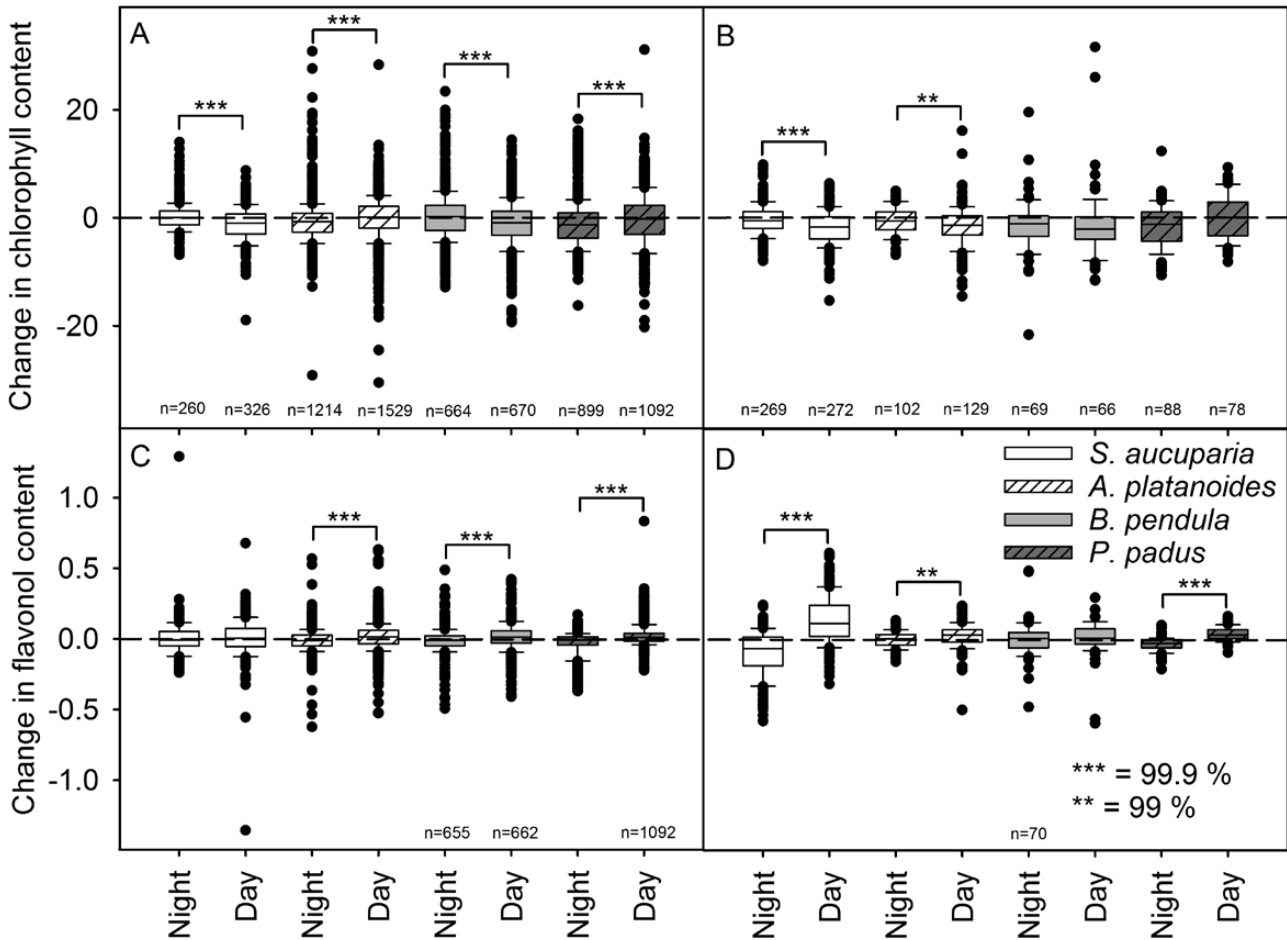
Figure 5. Changes in chlorophyll (A, B) or flavonol (C, D) contents during the nights (Night) or during the days (Day) in *Sorbus aucuparia* (white boxes), *Acer platanoides* (white hatched boxes), *Betula pendula* (grey boxes) and *Prunus padus* (dark grey hatched boxes) leaves. The changes are calculated for all pairs of two subsequent measurement points, separately from leaflets (*S. aucuparia*) or leaves (other species) with steady chlorophyll level (A, C) and from leaflets or leaves in which chlorophyll is being rapidly degraded (B, D). The boxes show 50 % of the data (the line indicates median), error bars show 10th and 90th percentiles, and outliers are drawn as dots. Asterisks indicate the cases where changes during the nights and changes during the days differ significantly with 99.9 % (***) or 99 % (**) probability, calculated with Mann–Whitney *U* test. Sample sizes are written below each box plot and they are similar for chlorophyll and flavonol measurements except in the case of *P. padus* in (C).

different, in this case suggesting chlorophyll degradation during the days (Fig. 5A). In *A. platanoides* and *P. padus* leaves with stable chlorophyll content, chlorophyll content seemed to slightly decrease during the nights (Fig. 5A).

In those *S. aucuparia* and *A. platanoides* leaves in which fast chlorophyll degradation had commenced, the loss of chlorophyll took place in the daytime (Fig. 5B). In contrast, in *P. padus* and *B. pendula* leaves with fast chlorophyll degradation, the chlorophyll content decreased similarly during the nights and the days (Fig. 5B). Loss of chlorophyll both during days and nights can also be seen in individual leaves of *B. pendula* and *P. padus* (Figs 3 and 4).

In *A. platanoides*, *B. pendula* and *P. padus* leaves with stable chlorophyll concentration, the flavonol content did not, on the average, change during the nights but increased during the days (Fig. 5C). Flavonol content increased during the days also in the leaves with decreasing chlorophyll content in all the species except that for *B. pendula* the difference was not statistically significant (Fig. 5D), suggesting that in general, flavonols are synthetized during the daytime.

Discussion

Chlorophyll is mainly degraded during the last week before abscission

The present data suggest that there are at least two ways to senesce: chlorophyll content of a leaf stays

high for the whole autumn and starts to decrease only 5–10 days prior to abscission (in *A. platanoides*, *B. pendula* and *P. padus*; Figs 2–4), or chlorophyll is slowly degraded during the whole autumn (in *S. aucuparia*; Fig. 1). Even in the second case, the rate of chlorophyll degradation increases 3–5 days before abscission of the leaflet. It has been proposed that the competence to senesce, in terms of the enzymatic machinery, might already be ready in green leaves (Andersson *et al.* 2004; Fracheboud *et al.* 2009), and that rapid senescence is advantageous for a tree (Fracheboud *et al.* 2009). Our data reveal that the loss of chlorophyll, a sign of the implementation of senescence in an individual leaf, takes only 1–2 weeks before abscission, even though the autumn lasts 1–3 months.

Depending on species, chlorophyll is degraded either during the day or at all times

The circadian cycle regulates many photosynthesis-related functions of plants (e.g. Noordally *et al.* 2013), including the expression of many genes of LHC (Kloppstech 1985; Xu and Johnson 2001). The present study suggests that in *S. aucuparia* and *B. pendula* chlorophyll decreases during the day rather than the night in those leaves in which rapid chlorophyll degradation had not yet commenced (Fig. 5A). The chlorophyll content of an individual leaf may fluctuate both up and down in the autumn because the chlorophylls are synthesized and degraded in an active leaf. Chlorophyll turnover is partially associated with the repair cycle of PSII (Feierabend and Dehne 1996; Lin *et al.* 2014), in good agreement with the finding that the turnover rate increases in high light (Beisel *et al.* 2010). The half-life of chlorophyll (64.1 h in mature wheat leaves; Stobart and Hendry 1984), is short enough to allow small circadian fluctuations of the chlorophyll content.

Chlorophyll is synthesized in the daytime in angiosperms (e.g. Pan *et al.* 2015) because protochlorophyllide oxidoreductase is active only in the light (for review, see Masuda and Takamiya 2004). Diurnal fluctuations in leaf chlorophyll content can also be caused by circadian oscillations in amounts or activities of other enzymes required in chlorophyll synthesis (Papenbrock *et al.* 1999). Our data confirm that fluctuations in chlorophyll contents of tree leaves are small (see Reddy *et al.* 2000).

Diurnal variation in the rate of chlorophyll degradation during senescence has not been earlier studied. It could be argued that it is beneficial for plants to degrade chlorophyll during the night when the phototoxic intermediates of chlorophyll degradation would be less dangerous. However, in *S. aucuparia* and in *A. platanoides*, more chlorophyll was lost during the days than during the nights (Fig. 5B) and in *B. pendula* and *P. padus* there

were no significant day/night differences in the rate of chlorophyll degradation (Fig. 5B). Thus, our data indicate that in spite of possible production of singlet oxygen during daytime, chlorophyll degradation is not confined to the dark period in the studied plants, but instead these species degrade chlorophyll in the light, probably relying on ROS-protection mechanisms like early light induced proteins (Andersson *et al.* 2004; Heddad *et al.* 2006).

Chlorophyll *a/b* ratio stays stable nearly to abscission time

In our data, chlorophyll *a/b* ratio stayed constant in the senescing leaves of all four trees of different species until chlorophyll was degraded to less than 10 µg Chl cm^{-2} [see **Supporting Information—Fig. S6**]. This is consistent with the hypothesis that plants keep the photosynthetic machinery functional until a very late stage of senescence (Keskitalo *et al.* 2005; Moy *et al.* 2015). When the chlorophyll content had reached the limit, the ratio decreased in *A. platanoides* and *S. aucuparia*. Decrease in the chlorophyll *a/b* ratio, resulting from slower degradation of chlorophyll *b* compared to *a*, has been observed in many senescing trees (Wolf 1956; Lee *et al.* 2003; Castro and Sanchez-Azofeifa 2008). Decrease of the chlorophyll *a/b* ratio may indicate that PSI is degraded faster than PSII (Andersson *et al.* 2004; Moy *et al.* 2015) or that LHCII stays intact longer than the reaction centres (Lepeduš *et al.* 2010; Moy *et al.* 2015). In our data, chlorophyll *a/b* ratio increased in *P. padus* [see **Supporting Information— Fig. S6**], which might be caused by higher stability of PSI compared to PSII, as is common in some annual species (e.g. Yamazaki *et al.* 1999). In senescing *Fagus sylvatica*, the chlorophyll *a/b* ratio first slightly increases and then decreases (Kraj 2015).

What is the role of enhanced flavonol synthesis?

In those *B. pendula* leaves that lost their chlorophyll around 1 October, flavonols increased more than in leaves that stayed green longer (Fig. 3), and light intensities during the later days were lower (see Table 1 and **Supporting Information—Fig. S1**). Also in *S. aucuparia*, rapid flavonol accumulation seemed to occur during bright days (Table 1), suggesting that high light enhances flavonol synthesis. However, the relationship between brightness of the day and flavonol synthesis did not hold for data collected during the early autumn (Table 1). Synthesis of flavonols also takes place in the daytime (Fig. 5C and D).

It has been previously shown in birch trees that certain flavonols increase in response to UV-B radiation (e.g. Morales *et al.* 2010). Because flavonols do not absorb UV-B as efficiently as other flavonoids (Pollastri

Table 1. Night and day temperature, total irradiance (sum) and maximum daily light intensity (max), and flavonol synthesis in rowan and silver birch leaves during selected days during the autumn. Increase in flavonols during the respective days is shown with symbols, where 0 represents no change in flavonol levels and +++ represents the highest observed increase.

Species		*T* (night), °C	*T* (day), °C	Light (sum), kJ m^{-2} day^{-1}	Light (max), kJ m^{-2} h^{-1}	Flv
Sorbus aucuparia	25.8	16	27	16 525	2300	+
	27.8	14	20	10 641	1447	++
	23.9	12	15	5469	868	+
	25.9	11	15	5060	778	+
Betula pendula	31.8	9	21	17 721	2146	0
	2.9	14	13	2766	270	+
	28.9	9	13	8737	1375	+++
	30.9	11	17	10 071	1631	+++
	19.10	1	11	6555	1130	+
	21.10	3	10	2956	742	++

and Tattini 2011), Majer *et al.* (2014) concluded that the differences in flavonol composition between sun and shade leaves of *Tilia platyphyllos* do not contribute to UV screening, but instead flavonols function as quenchers of singlet oxygen in strong visible light. Due to the short diffusion distance of singlet oxygen (for review, see Mattila *et al.* 2015), only flavonols present in the cytoplasm may efficiently protect the cells by reacting with this harmful species. Chlorophyll degradation may enhance flavonol synthesis because leaves that contain less chlorophyll are more efficiently illuminated throughout the leaf tissue than heavily pigmented leaves. Enhancement of photoinhibition of PSII by loss of chlorophyll (Pätsikkä *et al.* 2002) is an example of an analogous situation.

Flavonols have also been shown to increase in response to cold in dwarf birch (Stark *et al.* 2015), and flavonol content correlated with cold tolerance in hybrid *Arabidopsis thaliana* (Korn *et al.* 2008). Here, when the first *S. aucuparia* leaves fell off, the weather was warmer than later in August; however, flavonols increased at least equally rapidly in the leaves that fell off first and in those lasting longer (Fig. 1 and Table 1). Furthermore, weather was not particularly cold when flavonols strongly increased in *B. pendula* in mid-autumn (Fig. 3 and Table 1), and no additional synthesis of flavonols was observed in non-senescing leaves in October when the night temperature fell below zero. However, more data would be needed to confirm the generality of these findings.

In addition to their suggested roles in ROS-scavenging, flavonols might also function in protection against pathogens or herbivores (Martemyanov *et al.* 2015; Yang *et al.* 2016). However, birch leaves with intense yellow colour have been shown to attract more aphids than greener leaves (Sinkkonen *et al.* 2012), and larvae feeding on birch leaves with a lower chlorophyll content are not more conspicuous to birds than larvae on control leaves (Koski *et al.* 2017). Some flavonols are toxic also to plants (e.g. Kandil *et al.* 2004) and flavonols of the falling leaves might suppress the growth of competitors under the tree.

Conclusions

It may take over 2 months for a tree to shed all its leaves during autumn senescence, but our data suggest that most of this time, individual leaves retain a relatively high chlorophyll content and the average chlorophyll content decreases only because some leaves lose chlorophyll rapidly. For that reason, a complete picture about tree senescence cannot be achieved by studying senescence as an averaged phenomenon. The rate of chlorophyll degradation, once it commences, is quite uniform in a tree. Thus, trees keep photosynthetically active leaves alongside with leaves from which nitrogen is rapidly remobilized before abscission. Constant slow decrease in chlorophyll content during the whole autumn observed in *S. aucuparia* suggests that senescence strategies vary with species. The present data also suggest that increase in flavonols is common during senescence, but their role still needs to be resolved.

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Contributions by the Authors

H.M., D.V., V.H., S.K., O.V. and M.A. did the experiments, K.B.M. provided help and guidance with methods and E.T. supervised the project. H.M. wrote the paper with contributions from all authors.

Conflict of Interest

None declared.

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Supporting Information

The following additional information is available in the online version of this article—

Table S1. The effect of the measurer (five persons) on the chlorophyll or flavonol (Flv) levels in the four species, calculated by ANOVA. A statistically significant difference is highlighted with ***.

Figure S1. Weather in Turku, Finland, 24 August–21 October 2015. The grey area shows total irradiance (kJ m^{-2} day^{-1}) and the black dashed line shows maximum daily light intensity (as kJ m^{-2} h^{-1}). Day temperature is calculated as an average temperature at 1200–1600 h (from 1 October onwards 1300–1600 h), and night temperature as an average temperature at 0400–0600 h (except before 1 August, 0200–0400 h and after 12 October 0500–0700 h). The data are from the Finnish Meteorological Institute.

Figure S2. Phenolic compounds detected with high-performance liquid chromatography (HPLC) at 355 nm from leaf extracts. (A) Chromatograms. (B–D) Spectra of all extracted compounds of *Betula pendula* (B), *Acer platanoides* (C) and *Sorbus aucuparia* (D). Retention times (RT) and classifications of the compounds are given.

Figure S3. Chlorophyll content in *Sorbus aucuparia* leaves during autumn senescence. At every measurement date, 10 randomized leaflets were collected and chlorophylls were extracted in *N,N*-dimethylformamide (DMF) and quantified spectrophotometrically. Mean values and error bars showing SD (A); individual measurements (B).

Figure S4. Senescing *Sorbus aucuparia* (A, B), *Acer platanoides* (C), *Betula pendula* (D) and *Prunus padus* (E) trees.

Figure S5. Rates of changes in chlorophyll contents from *Sorbus aucuparia* (A), *Acer platanoides* (B), *Betula pendula* (C) and *Prunus padus* (D) leaves plotted against rates of changes in flavonol contents. Rates during 2.5 days are calculated as slopes of lines from slightly smoothed data.

Figure S6. Chlorophyll *a* to *b* ratio as a function of total chlorophyll concentration of the sample in the four species, measured during different phases of autumn senescence. Chlorophylls were extracted in *N,N*-dimethylformamide (DMF) and quantified spectrophotometrically.

Figure S7. Comparison of leaf chlorophyll content measured with a destructive method by extracting pigments in *N,N*-dimethylformamide (DMF) or with non-destructive measurements from *Sorbus aucuparia* (A), *Acer platanoides* (B), *Betula pendula* (C) and *Prunus padus* (D) leaves, collected at different dates during autumn.

Figure S8. The total amount (relative units) of all compounds detected with high-performance liquid chromatography (HPLC) (circles) or those compounds identified as flavonols by their spectra (squares), from extracts of *Sorbus aucuparia* leaflets (A), *Acer platanoides* leaves (B) and *Betula pendula* leaves (C), compared to the flavonol index measured from the same leaves with Dualex.

Figure S9. Absorbance at 355 nm, reflecting the amount of each individual compound detected with high-performance liquid chromatography (HPLC) after extraction of phenolics from leaves of *Betula pendula* (A), *Acer platanoides* (B) and *Sorbus aucuparia* (C) compared to the flavonol index measured from the same leaves with Dualex. The compounds are classified to six groups. The numbers indicate the chlorophyll content of the sample as µg Chl m^{-2}.

Figure S10. Effect of ambient light (A, B) and handling (C) on the measuring accuracy of Dualex. (A, B) Chlorophyll and flavonol contents were measured from the same *Acer platanoides* and *Prunus padus* leaves in the presence of different amounts of incident light (photosynthetic photon flux density, PPFD, of 0–100 µmol m^{-2} s^{-1}). (C) Chlorophyll (hatched bars) and flavonol (grey bars) contents of *P. padus* leaves were measured in darkness so that the measuring heads of Dualex were either kept loosely together or pressed strongly together. The averages (*n* = 6) are significantly different with 99.9 % (***) or 95 % (*) probability, calculated with Student's *t*-test. The error bars show SD calculated from 3–6 technical repetitions and they are shown when larger than the symbols.

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Appendix 1: Validation of the Optical Method for Chlorophyll and Flavonol Measurements from Senescing Leaves

To validate chlorophyll measurements from senescing leaves with a fluorescence and transmittance based sensor (Dualex Scientific™), we compared the chlorophyll content measured with the non-destructive method with extraction of chlorophylls in *N,N*-dimethylformamide (DMF). The results obtained with the two methods showed linear correlation (R^2 = 0.8–0.96) in a wide range of chlorophyll concentrations **[see Supporting Information—Fig. S7]**. In agreement with earlier literature (Cerovic *et al.* 2012; Casa *et al.* 2015; Agati *et al.* 2016), species-specific calibration was found to be necessary for quantitative measurements **[see Supporting Information—Fig. S7]**.

To validate the non-destructive flavonol measurements from senescing leaves, flavonols were also measured with high-performance liquid chromatography (HPLC) **[see Supporting Information—Fig. S8]**. In the case of senescing leaves, a good correlation between the optical method and HPLC was obtained in *Betula pendula* leaves, although the relationship is somewhat curvilinear **[see Supporting Information—Fig. S8C]**. The reason for the good correlation in *B. pendula* might be that this species does not synthetize anthocyanins in contrast to *Sorbus aucuparia* and *Acer platanoides*. Accordingly, large amounts of phenolic acids and non-flavonol flavonoids were observed in *S. aucuparia* and *A. platanoides*, and many of these compounds also increased during senescence **[see Supporting Information—Fig. S9]**, but the optical method correlated with both the amount of flavonols and with the total amount of phenolic compounds **[see Supporting Information—Fig. S8]**. A good correlation with the amounts of flavonols in the leaf measured with the optical method and with HPLC is in accordance with previous research (Barthod *et al.* 2007; Pfündel *et al.* 2007; Agati *et al.* 2016), although the optical method is expected to measure epidermal compounds, whereas extraction methods measure an average of the whole leaf. However, flavonols cannot be distinguished from other phenolic compounds with the optical method. In our case, this difficulty has only minor importance because relative differences in the amounts of flavonols in leaves at different stages of senescence were highly correlated with relative differences in the amounts of all phenolic compounds **[see Supporting Information—Fig. S8]**.

We conducted the measurements in field conditions, either at sunrise or at sunset, both defined by the position of half of the Sun above the horizon. Ambient light was found not to affect chlorophyll measurements **[see Supporting Information—Fig. S10A]**. Flavonol values showed some variability with ambient light **[see Supporting Information—Fig. S10B]**, and high incident light (photosynthetic photon flux density ~100 μmol m^{-2} s^{-1}) occasionally caused an inability to record any value. However, no consistent dependence of the flavonol signal on ambient light was observed.

We found that pressing the measuring heads of the Dualex device more firmly together slightly lowered the observed chlorophyll value and increased the flavonol value **[see Supporting Information—Fig. S10C]**, suggesting that the results might depend on the measurer. However, according to ANOVA analysis, the measurer (five persons here) did not have a significant effect on the chlorophyll or flavonol levels, except for the case of the chlorophyll content of *A. platanoides* **[see Supporting Information—Table S1]**. Even though the assumptions of ANOVA are not met in the present data, the result suggests that the effect of the measurer is small.