Tree age-related effects on sun acclimated leaves in a chronosequence of beech (*Fagus sylvatica*) stands

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Abstract. The assessment of the effect of tree age on leaves is usually limited by the difficulty of sampling sun leaves from tall ageing trees. In this study, we investigated tree age-related effects on sun leaves in a chronosequence of beech (*Fagus sylvatica* L.) stands. The effects of stand age on leaf mass to area ratio (LMA), chlorophyll (Chl), epidermal polyphenols (EPhen), nitrogen and carbon contents in sun leaves were investigated in 17 even-aged stands distributed into six age classes (14–175 years old). Chl and EPhen were assessed *in vivo* with SPAD and Dualex portable leaf-clips respectively. Leaves were sampled by shooting and sun leaves were identified based on criteria obtained from a vertical profile of the ratio abaxial vs adaxial EPhen across the canopy. Sun leaves were characterised by a high and similar adaxial and abaxial EPhen contents, high LMA value and low mass-based Chl content. These sun leaf characteristics, together with leaf nitrogen and carbon contents, were not significantly affected by stand age. Along the chronosequence, beech trees invested a stable fraction of leaf mass into nitrogen, carbon, Chl and EPhen with decreasing leaf size, i.e. dry mass and area.

Introduction

In temperate deciduous forests, stands typically develop with increasing leaf area in the canopy and increasing productivity from an early stage to maturity, followed by a gradual decrease in leaf biomass and whole-tree productivity with ageing (Gower *et al*. 1996; Nock *et al*. 2008; Genet 2009). During tree development, changes in resource availability (Ryan *et al*. 2004; Legout *et al*. 2008) and alterations in resource allocation and partitioning within trees (Ryan *et al*. 1997; Genet *et al*. 2010) may affect, leaf morphology, structure, phytochemistry and function. A decrease of leaf nitrogen (N) and chlorophyll (Chl) contents (Martínez-Vilalta *et al*. 2007) and leaf mass per area ratio (LMA) (Thomas and Winner 2002; Delzon *et al*. 2004) contribute to reduce photosynthetic activity in ageing trees, in addition to height-related hydraulic limitations (Delzon *et al*. 2004; Ryan *et al*. 2006; Martínez-Vilalta *et al*. 2007). Increasing seed production with tree age may influence leaf characteristics as well (Gross 1972; Innes 1994; Thomas 1996; Leal and Thomas 2003). However, most of these studies have focussed on conifers; tree age-related effects on leaf characteristics are poorly

documented in temperate deciduous species. Investigating tree age effects on leaf traits is difficult due to sampling constraints in tall trees and the effects of exogenous covariates such as changes in light environment within the canopy, nutrient availability, severe climatic and biotic events and forest management practices. Investigations into the effects of tree age on leaf traits need to be standardised at least by sampling only sun acclimated leaves from the upper canopy.

Acclimation of leaves to irradiance is usually described through the vertical profiles of LMA and mass-based chlorophyll content (Chl_m) within the canopy (Niinemets 1995; Niinemets and Tenhunen 1997; Niinemets *et al*. 2004; Montpied *et al*. 2009). Leaf thickness and density and, therefore, LMA, increase from the base to the top of the canopy (see for a review Poorter *et al*. 2009). Mass-based nitrogen content (N_m) is usually constant across the canopy, but from base to top of the canopy leaf N is more invested into Rubisco than into chlorophyll binding proteins; therefore, Chl_m and the ratio between chlorophyll and nitrogen (Chl/N) decreases (Niinemets and Tenhunen 1997; Niinemets *et al*. 2004; Montpied *et al*. 2009).

In addition, leaves acclimate to full sunlight by accumulating polyphenols, mainly soluble glycosylated flavonoids in epidermal vacuoles (Searles *et al*. 2001; Bidel *et al*. 2007). Epidermal polyphenols (EPhen) are an efficient sunscreen, protecting the mesophyll against incident ultraviolet (UV) radiation (Caldwell *et al*. 1983). The amount of EPhen accumulated in the adaxial and abaxial epidermis depends on the UV irradiance on each leaf side, which, in turn, is controlled by leaf inclination, increasing from base to top canopy (Tattini *et al*. 2005; Bidel *et al*. 2007; Planchais and Sinoquet 1998). Therefore, in addition to LMA, Chl_m and Chl /N, the ratio of abaxial : adaxial EPhen is probably an efficient indicator of leaf exposition to local irradiance in beech.

Non invasive sensing of Chl and EPhen in the field at different stand ages may provide new insights into tree age-related changes in carbon (C) and nitrogen economy within leaves. Leaf carbon and nitrogen economy are reflected by mass investments into polyphenol and protein synthesis (Jones and Hartley 1999). In contrast with proteins, polyphenols are C-rich and N-free molecules (Poorter 1994). They include flavonoids, hydroxycinnamic acids, tannins and lignins that are involved in plant structure and protection (Jones and Hartley 1999). In leaves of woody species, ~4–20 and 5–12% of carbon is invested into lignins and soluble phenolics (like glycosylated flavonoids) respectively. This relative allocation varies according to species and environment (Poorter 1994; Poorter and Villar 1997). In leaves, up to 80% of the nitrogen is invested into photosynthetic proteins (Evans 1989), a fraction of which (~20–30%, Hikosaka and Terashima 1995) is bound to Chl. The synthesis of proteins and of polyphenols, except for a fraction of hydrolysable tannins, compete for the use of a common precursor, the amino acid L-phenylalanine (PHE), which is assumed to be limiting (Jones and Hartley 1999). The synthesis of proteins and polyphenols are, therefore, two competing metabolic pathways. The allocation to PHE depends on leaf development stage, resource availability (light, nutrients) and biotic attacks (Jones and Hartley 1999). In mature leaves, nutrient limitation, biotic attack and high light stimulate the production of polyphenols at the expense of proteins, whereas high nutrient levels and low light availability promote protein synthesis at the expense of polyphenols (Jones and Hartley 1999). Therefore, the ratio of proteins to polyphenols provides an indication of the nitrogen over carbon content in leaves. Chl and EPhen can be used as surrogates of proteins and polyphenols, respectively (Meyer *et al*. 2006) and, therefore, variations of the Chl/EPhen ratio can be related to variations of the N/C ratio (Louis *et al*. 2009).

This study aimed to assess tree-age effects on sun leaf traits in a chronosequence of beech stands (*Fagus sylvatica*). Our specific objectives were to:

(i) define a new indicator for sun leaves based on the vertical profiles of the ratio of abaxial : adaxial EPhen; and
(ii) investigate changes in the traits of sun-acclimated leaves along a chronosequence; sun leaves being identified among the sampled leaves, from the ratio of abaxial : adaxial EPhen.

Materials and methods

Plant material and experimental design

Two experiments were performed with beech (*Fagus sylvatica* L.) trees in the field at two different locations. Experiment 1 (Exp1) aimed to assess the vertical profile of abaxial : adaxial EPhen in a 30-year-old beech stand. Exp1 was a prerequisite for the identification of sun leaves during the sampling in the chronosequence of Experiment 2 (Exp2).

Exp1 was performed at the experimental long-term ecosystem monitoring site of Hesse, North Eastern France (48°45′N, 6°20′E, mean elevation 255 m), in a young beech stand. Stand characteristics are described in detail by Granier *et al*. (2002). Four campaigns of measurements were made along a vertical profile in the canopy of 30-year-old beech trees throughout the summer of 2001 (10–22 June, 17–20 July, 7–10 August and 28–30 August). During each campaign, ~3–4 fully expanded leaves were sampled at six different heights (~21 leaves per campaign), and three SPAD and two Dualex measurements (abaxial and adaxial faces) were performed on each leaf. Leaf inclination was visually classified according to three classes: planophile, erectophile and intermediate. Leaves were then sampled for Chl content and area and dry mass recorded (85 leaves total per sampling). LMA (leaf mass to area ratio) was used as a surrogate for the irradiance received by leaves during their growth (Niinemets and Tenhunen 1997; Montpied *et al*. 2009).

Exp2 was performed in the state forest of Fougères, North Western France (48°23′N, 1°09′W, mean elevation 164 m). A chronosequence of 25 beech stands was selected to display similar climatic, edaphic and management conditions (Genet *et al*. 2010). The chronosequence was composed of pure even-aged, naturally regenerated stands, located on Alocrisols-Neoluvisols (Baise and Girard 1998). The 25 stands were distributed into six age classes (average ages: 14, 35, 58, 95, 138 and 175 years, 3–4 stands per class). Ten dominant trees were sampled per stand during late June 2006, when leaves were fully expanded and mature. On each tree, one (rarely two) south-exposed branch of the upper third canopy was severed using a shotgun in the adult stands or a branch-lopper in the youngest stands (Genet 2009). A total of 30 fully expanded leaves was harvested, among which 10 fully expanded leaves was sampled per tree for SPAD, Dualex and Chl measurements. Ten SPAD and 4–10 Dualex measurements were performed per leaf side. Dualex measurements were monitored in 17 stands over the total 25 stands. The 30 leaves were used to record LMA, carbon and nitrogen content. In the field, the 30 leaves were stored in a cooler containing freezer packs. In the laboratory, the samples were stored at 4°C until measurements were made.

In vivo *optical assessment of leaf Chl and EPhen contents*

Chl and EPhen were measured in the field using two portable leaf clips meters. Leaf Chl was assessed using the SPAD-502 Chl meter (Konica-Minolta, Carrière-sur-Seine, France). A calibration curve was established using Chl measurements of leaf extracts (see below), and the SPAD values were converted into area-based Chl content (Chl_a). The mass-based Chl content, Chl_m, was obtained by dividing Chl_a by LMA.

EPhen were estimated using the Dualex portable leaf-clip (Force-A, Orsay, France) according to Cartelat *et al*. (2005).

Dualex measures the UV absorbance of the leaf epidermis by double excitation of Chl *a* fluorescence (Bilger *et al*. 1997), using UV (375 nm) and red (650 nm) light, as described earlier (Goulas *et al*. 2004). Epidermal UV-absorbance is determined from the UV/red Chl fluorescence excitation ratio. Red light is not absorbed by the epidermis and reaches the mesophyll where it excites Chl. Therefore, red-excited Chl fluorescence can serve as a reference signal to which UV-excited Chl fluorescence can be related. Absorbance at 375 nm is due mainly to water-soluble flavonoids stored in epidermal vacuoles (Cerovic *et al*. 2002). The prototype version and the commercial version (Dualex3) of the Dualex leaf-clip were used in Exp1 and Exp2 respectively. The Dualex readings of leaf adaxial and abaxial sides, $EPhen_{ad}$ and $EPhen_{ab}$, respectively, were summed to estimate the total area-based EPhen content ($EPhen_a$). Mass-based EPhen content ($EPhen_m$) was obtained by dividing $EPhen_a$ by LMA. In woody species like beech, the main absorbers detected at 375 nm are flavonols, which could be involved in lignification (Bate-Smith 1962; Louis *et al*. 2009). Quercetin derivates are the most universally distributed flavonoids (Bate-Smith 1962). Therefore, $EPhen_a$ is expressed in molar units of quercetin 3-O-glycoside (quercitrin) equivalents calculated from the Dualex-derived absorbance according to Louis *et al*. (2009). Chl_m and $EPhen_m$ represent the proportion of leaf dry mass allocated to Chl and EPhen, respectively. By calculating the Chl/EPhen ratio, an estimate of the relative fraction of leaf dry mass invested into Chl and EPhen was obtained.

Leaf chlorophyll extraction and leaf mass per area

The calibration of the two SPADs used and the transformation of SPAD units into that of Chl in $g\, cm^{-2}$ were performed according to the method by Barnes *et al*. (1992). Leaf area was measured within 48 h following harvesting. Leaf area was measured using a planimeter LI-3000A with a transparent belt conveyer LI-3050A (Li-Cor Inc., Nebraska, NE, USA). Leaves were then oven-dried for 72 h at 60°C before being weighed. The LMA was the ratio of cumulated dry weight to leaf area ($g\, m^{-2}$). The three values were then averaged by tree.

Nitrogen and carbon content

After drying, the 30 leaves per tree were ground using a rotary grinder with rings (SODEMI, Cergy Pontoise, France). Before analysis, samples were dried again for 4 h at 60°C. An aliquot of 1.5–1.7 mg of powder was weighed on a Sartorius MC5 microscale (Data Weighing Systems Inc., Chicago, IL, USA), stored in a tin capsule and analysed by dynamic flash combustion with an elemental analyser (Flash 2000 NC, Thermo Fischer Scientific Inc., Miami, FL, USA).

Data analysis

An *a posteriori* subsampling was done in Exp2 in order to select only full-sun acclimated leaves. Based on Exp1, Louis *et al*. (2009) and Meyer *et al*. (2009), the criterion of selection was that the value of the ratio of $EPhen_{ab}/EPhen_{ad} > 0.8$. If one of the 10 sampled leaves of a given tree did not meet the criterion, the tree was removed from the stand sampling. This selection discarded 4–9 trees per stand and five among the 17 stands. $EPhen_{ad}$,

$EPhen_{ab}$ and LMA were normalised to the maximum to allow comparison between the two experiments.

Statistical analysis was conducted with Igor PRO 6.03 (WaveMetrics Inc., Lake Oswego, OR, USA) and Statistica 6.1 (Statsoft Inc., Maison-Alfort, France). In Exp1, effects of measurement campaign and of sampling height on LMA, Chl_m, $EPhen_{ad}$ and $EPhen_{ab}$ were tested using a two-way analysis of variance (ANOVA) on log-transformed data. This analysis did not reveal any effect of the date of measurement on LMA ($n = 69$, d.f. = 3 and 65, $F = 1.17$, $P < 0.33$), Chl_m ($n = 68$, d.f. = 3 and 64, $F = 2.19$, $P < 0.099$), $EPhen_{ad}$ ($n = 69$, d.f. = 3 and 65, $F = 2.12$, $P < 0.11$) and $EPhen_{ab}$ ($n = 69$, d.f. = 3 and 65, $F = 0.56$, $P < 0.64$). Therefore, we based our analysis on the mean of all the sampled leaves from the four measurement campaigns. In Exp1, the difference between $EPhen_{ad}$ and $EPhen_{ab}$ at each sampling height was tested using a Student's *t*-test at the 0.01 significance level. Stand age effects on LMA, leaf area, leaf dry mass, area-based nitrogen content (N_a), N_m, Chl_a, Chl_m, Chl/N, $EPhen_{ad}$, $EPhen_{ab}$, $EPhen_a$, $EPhen_m$ and the Chl/EPhen ratio were tested in Exp2 from a regression analysis. The relationships between optical and biochemical parameters in Exp2 were examined with Pearson's correlation analysis.

Results

Vertical profile of EPhen and Chl contents in Exp1

Fig. 1 shows the vertical profile of LMA, Chl_m, $EPhen_{ad}$ and $EPhen_{ab}$ from the base to the top of the canopy of the 30-year-old beech trees. Leaves were planophile at the base and almost erectophile at the top of the canopy (data not shown). LMA significantly increased ($n = 69$, d.f. = 5 and 63, $F = 313.9$, $P < 0.001$) and Chl_m significantly decreased ($n = 68$, d.f. = 5 and 62, $F = 85.46$, $P < 0.001$) with height. $EPhen_{ad}$ and $EPhen_{ab}$ significantly increased ($n = 69$, d.f. = 5 and 63, $F = 161.0$, $P < 0.001$ and $n = 69$, d.f. = 5 and 63, $F = 341.7$, $P < 0.001$, respectively) with height, especially in the upper half of the crown. $EPhen_{ab}$ was significantly lower than $EPhen_{ad}$ along the profile, but they converged to a very close and high value at the top of the crown. Full sun leaves at canopy top displayed the highest LMA, $EPhen_{ad}$ and $EPhen_{ab}$ and the lowest Chl_m.

Estimation of leaf acclimation to light at different stand ages

During Exp1, we found positive relationships between normalised epidermal phenolic contents ($EPhen_{ad}$ and $EPhen_{ab}$) and LMA (Fig. 2). The values of $EPhen_{ad}$ and $EPhen_{ab}$ measured across the chronosequence (Exp2) were distributed along the same relationships over a range of values corresponding to the upper quarter of the canopy (see Fig. 1, 12–17 m height). In this canopy layer, $EPhen_{ad}$ reached saturation and $EPhen_{ab}$ increased sharply as the leaves switched from horizontal to vertical in full sun acclimated leaves. This comparison between the two experiments leads to the conclusion that leaves sampled in Exp2 were not uniformly full-sun acclimated. Such a sampling would lead to a bias due to combined effects of relative irradiance and stand age. An *a posteriori* subsampling selected only full-sun acclimated leaves. After this subsampling, $EPhen_{ab}$ in Exp2 (Fig. 2*b*) was distributed
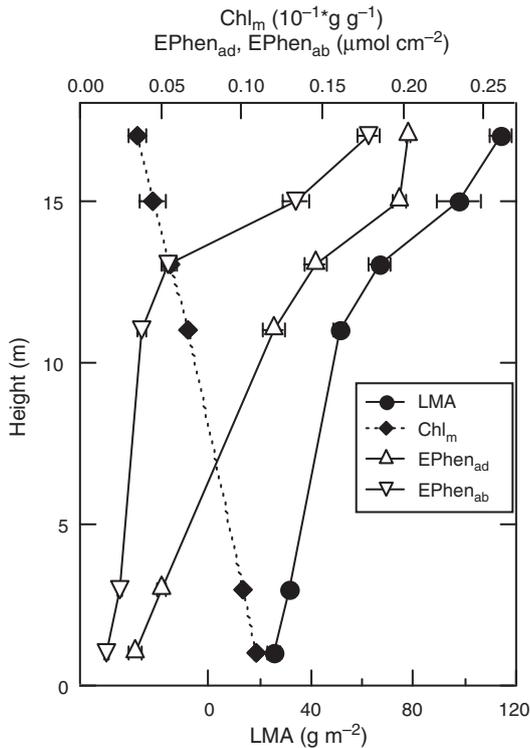
Fig. 1. Relationship between leaf mass per area (LMA), mass-based Chl content (Chl$_m$), area-based adaxial and abaxial EPhen content (EPhen$_{ad}$ and EPhen$_{ab}$ respectively) and height (m) in a beech canopy in Exp1. Mean ±s.e. (n = 11–17 leaves).

over a range of values corresponding to the top of the canopy (see Fig. 1, 14.5–17 m height).

Variations in sun leaf traits across the chronosequence

In the subsample, LMA of sun leaves did not vary and remained high throughout the chronosequence (Fig. 3*a*). Leaf area and leaf dry mass decreased markedly and similarly along the chronosequence (Fig. 3*b*).

The other leaf characteristics, that is, nitrogen, carbon, Chl and EPhen content did not vary across the chronosequence (Figs 4–6). Consequently Chl /N (Fig. 5) and Chl/EPhen (Fig. 6) remained stable across the chronosequence.

Relationship between optical and biochemical measurements

In Exp2, the significant positive relationships between Chl$_a$ and N$_a$ and between Chl$_m$ and N$_m$ (Table 1) show that, even among sun leaves, the mass invested in Chl increased with leaf nitrogen content. By contrast, there was no significant relationship between EPhen$_a$ and LMA and between EPhen$_a$, EPhen$_m$ and N$_m$ (Table 1); the mass invested in EPhen did not significantly decrease with leaf nitrogen. This was expected since EPhen$_a$ and EPhen$_m$ were highly stable across the chronosequence (Fig. 6*b*). Hence, the positive correlation between Chl/EPhen and N$_m$ or N/C was mainly ascribed to the positive relationship between Chl$_m$ and N$_m$ but the variations of Chl$_m$ and N$_m$ were not significant across the chronosequence (Figs 4, 5).

Discussion

Vertical profile of EPhen

EPhen$_{ad}$ and EPhen$_{ab}$ very closely described the acclimation of beech leaves along a vertical gradient of irradiance within the canopy. Leaves acclimate asymmetrically to the light microenvironment and the accumulation of EPhen in each leaf side is driven by the UV dose received by each epidermis (Grammatikopoulos *et al*. 1999; Bidel *et al*. 2007). UV radiation, especially UV-B, combined with visible light, is known to induce the synthesis of flavonols in the epidermis (Hollósy 2002; Bidel *et al*. 2007; Morales *et al*. 2010). From the base to the top of the beech canopy, predominant leaf inclination from the horizontal increased towards the vertical as reported by Larcher (2003) for *Quercus-Tilia*, and by Planchais and Sinoquet (1998) for beech saplings. The abaxial leaf side gradually receives more direct sunlight. At the top of the crown, in more vertical leaves, both leaf sides are similarly exposed to incident sunlight and therefore display similar area-based EPhen.

The vertical profiles of EPhen$_{ad}$ and EPhen$_{ab}$ are curvilinear and it has been shown that their sum (EPhen$_a$) is tightly linked with LMA (Meyer *et al*. 2006). EPhen synthesis in each epidermis was strongly related to leaf structure. Yamasaki and Kikuzawa (2003) also reported a curvilinear increase of LMA, total polyphenols and tannins in leaves of *Fagus crenata* along a vertical irradiance gradient in the canopy. They showed that leaf toughness increased in the canopy as leaf herbivory decreased. They concluded that there was an increase in carbon investment in polyphenols in sun leaves that were better protected against herbivores. The Dualex device cannot detect tannin and lignin that are strongly related to leaf toughness and defence, but it detects flavonols in the epidermis that are mainly involved in UV screening. A steady high UV screening in sun leaves is expected to correspond to a full acclimation to the local UV microclimate since EPhen content is mainly stable and not labile in mature sun leaves (Louis *et al*. 2009). In Exp1, EPhen$_{ad}$ saturated with increasing height as the increase of LMA and EPhen$_{ab}$ was dampened. According to Poorter *et al*. (2009) numerous factors could limit the rise of LMA at the top of crown. Here, the similarity of the profiles of EPhen (especially EPhen$_{ab}$) and LMA may result from the complex interactions between epidermal flavonols and auxin that control leaf architecture and therefore LMA (Brown *et al*. 2001; Jansen *et al*. 2001).

Stand age effect on sun leaf traits

A comparison between Exp1 and Exp2 revealed the similarity of vertical profiles of EPhen$_{ab}$ independently of stand age. According to the leaf characteristics (LMA, Chl, EPhen), leaf acclimation to full sunlight did not depend on tree age in beech. Each sampled branch in a stand comprised a mixture of leaves with various inclinations. This could be related to the clustering of erect leaves on short shoots, which induces mutual shading within sunny branches (Planchais and Sinoquet 1998). However, the proportion of subsampled trees with sun leaves decreased from 38% in young (14–50-year-old stands) to 12.5% in ageing (58–175-year-old stands) stands (see Fig. 3*a*). The proportion of subsampled trees would depend on the threshold value of the EPhen$_{ab}$/EPhen$_{ad}$ ratio. The vertical profile of EPhen$_{ab}$ is very steep, therefore, a small decrease in this ratio would include more
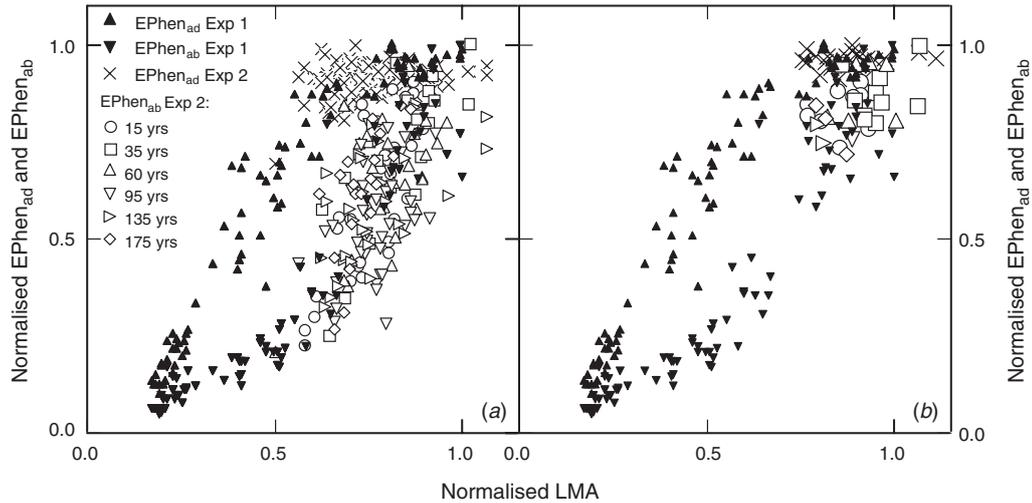
Fig. 2. Relationships between area-based adaxial and abaxial EPhen content (EPhen$_{ad}$ and EPhen$_{ab}$, respectively) and LMA in Exp1 and Exp2. The sampling (*a*) and the subsampling (*b*) of Exp2 are shown. For Exp1, one point corresponds to a single leaf. For Exp2, one point corresponds to a single tree from a given stand age. (*a*, *b*) In Exp1, EPhen$_{ad}$, EPhen$_{ab}$ and LMA were normalised to 0.21, 0.21 μmol cm^{-2} and 113 g m^{-2} respectively. (*a*, *b*) For Exp2, EPhen$_{ad}$, EPhen$_{ab}$ and LMA were normalised to 0.16, 0.16 μmol cm^{-2} and 110 g m^{-2} respectively. (*a*, *b*) Extremely high values of LMA were avoided for normalisation in Exp2.
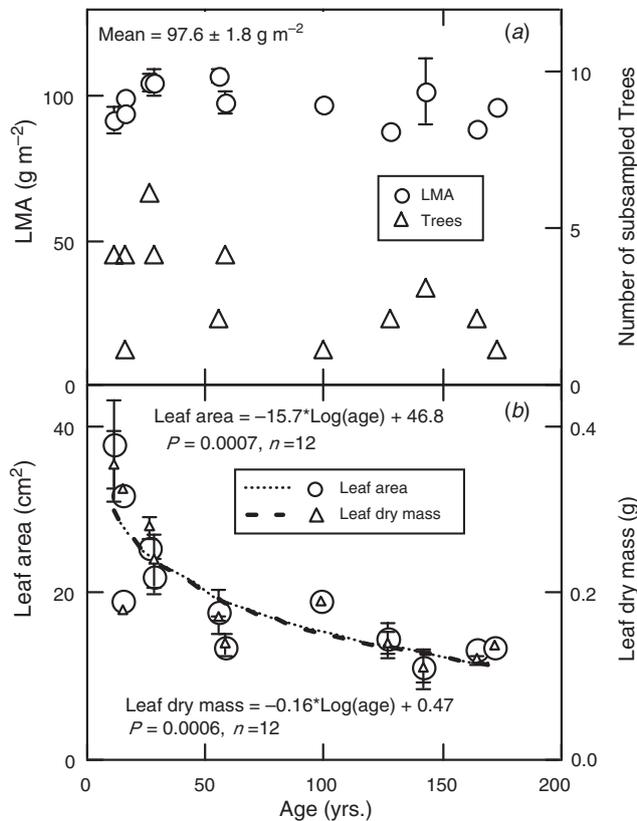

Fig. 3. Variation of (*a*) LMA and the number of subsampled trees and (*b*) leaf area and leaf dry mass across the chronosequence of beech stands in Exp2. One point corresponds to one stand (1–6 subsampled trees per stand). Error bars represent s.e. (*a*) Mean LMA (±s.d.) across the chronosequence is indicated (*n* = 12). (*b*) Regression equations are indicated.

subsampled trees, but with less uniformly sun acclimated leaves. Ideally, the ratio would be 1, that is, a similar steady-state value of EPhen$_{ad}$ and EPhen$_{ab}$, as shown by Louis *et al*. (2009) and Meyer *et al*. (2009) in cultivated young trees, but, in Exp2, this value was not reached in any of the studied stands. In Exp2, the threshold of 0.8 appeared to be the best compromise to subsample sun acclimated leaves along the chronosequence; below this value, EPhen$_{ad}$ was not at the plateau. The low proportion of subsampled trees revealed either an increasing difficulty in sampling sun branches by shotgun in the tallest trees or a change in branch architecture during stand development (Nicolini 1997; Nicolini and Chanson 2000) in such a way that self-shading increased among clustered leaves, as shown by Niinemets *et al*. (2005) for *Agathis australis*. The potential variation in sunny branch architecture during beech ageing was not measured in Exp2 and is unknown.

By subsampling the data according to sun leaf criteria, two age-related effects on leaf characteristics were clearly detected. First, in the youngest beech trees (<25 year-old stands), from which leaves were the most accurately sampled because of the small tree height, only 30% of the sampled leaves met sun leaves characteristics. Moreover, before subsampling, Chl$_m$ was the highest in this stand age class (see Table S1 stand 2, available as Supplementary Material to this paper). This suggests that leaf construction in young beech trees could be less plastic than in mature trees and that young beech trees produced leaves with more sciaphytic traits, in agreement with Niinemets (2006). This suggests also an effect of the crown architecture (Nicolini 1997); mean LAI was highest in the youngest beech trees (Genet *et al*. 2010). Second, in adult tall trees (95–175 years old), only 10% of the sampled leaves were sun leaves. They were more than 2-fold smaller and lighter than leaves from the youngest trees, but with similar LMA and mass investment in nitrogen, carbon, Chl and EPhen. Therefore, there was a reduction in leaf size in adult tall
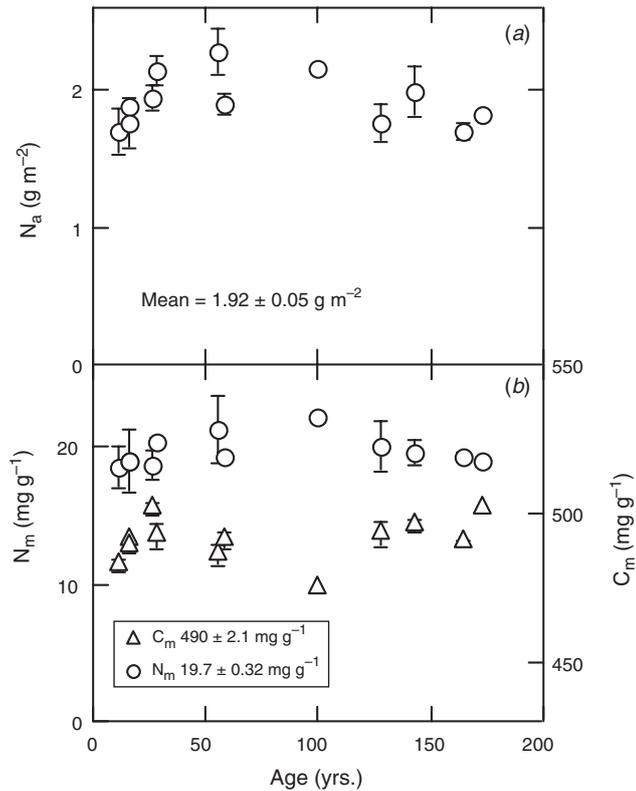
Fig. 4. Variation of (*a*) area-based leaf nitrogen (N_a) and (*b*) mass-based leaf nitrogen (N_m) and carbon (C_m) contents throughout the chronosequence of beech stands in Exp2. One point corresponds to one stand (1–6 subsampled trees per stand). Error bars represent s.e. Mean values (±s.d.) across the chronosequence are indicated (*n* = 12).

trees, but the pattern of the leaf structure and chemical composition remained constant. We can conclude that there is a proportional microphylly in old trees. However, we cannot conclude that these small leaves were produced in response to increasing hydraulic limitation at the top of ageing trees (Ryan *et al.* 2006; Drake *et al.* 2010), because LMA did not increase as usually observed in response to water limitation (Abrams 1994; Hallik *et al.* 2009). Further, the microphylly might be related to seed production (Innes 1994). During the masting year 1990, Innes and Boswell (1991) demonstrated a slight negative correlation between seed abundance and leaf size of beech. The year of our experiment (2006) was also a masting year with an important seed production in old stands (Genet *et al.* 2010). Moreover, Genet *et al.* (2010) reported a decrease in LAI from stand maturity onward. It is known that beech masting increases crown transparency in the current year (Gross 1972; Innes 1994). Han *et al.* (2008) showed that more nitrogen and carbohydrates were expended in producing buds containing leaf and flower primordials than in producing buds containing leaves only. Thus, a greater competition for resources between flower and foliar primordials might result in a reduction in leaf size. This competition effect on leaf size is well known in non-ligneous species and has been interpreted as a reduction in the leaf expansion rate or duration of expansion (Granier and Tardieu 2009). Most of the sampled leaves (90%) from aging trees were
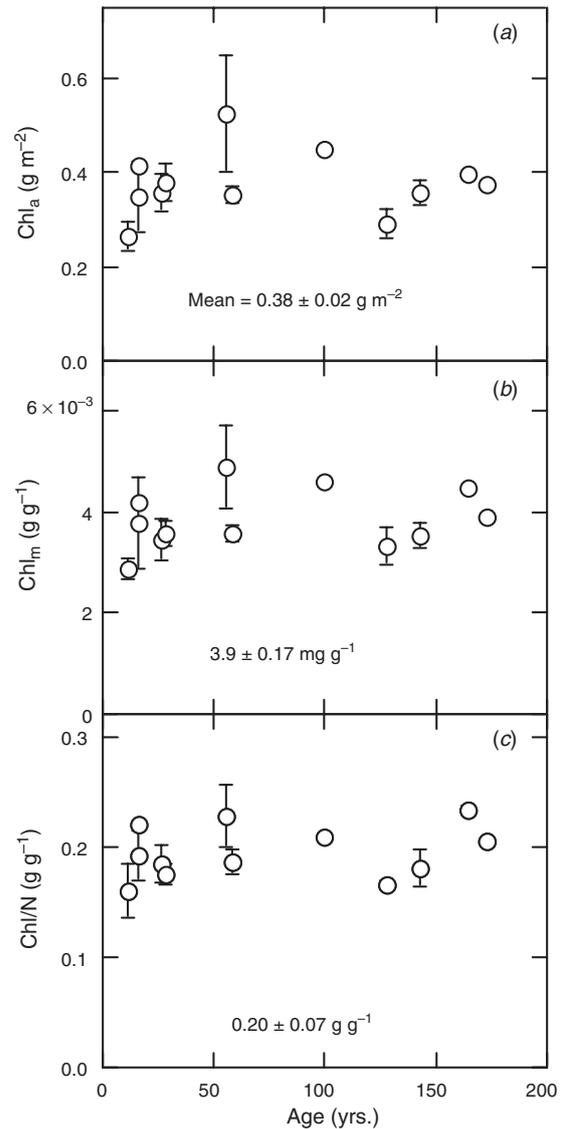

Fig. 5. Variation of (*a*) area-based Chl content (Chl_a), (*b*) mass-based Chl content (Chl_m) and (*c*) the Chl over nitrogen ratio (Chl /N), throughout the chronosequence of beech stands in Exp2. (*a*) Area-based data obtained from optical measurements; (*b*) mass-based data obtained after dividing area-based data by LMA. One point corresponds to one stand (1–6 subsampled trees per stand). Error bars represent s.e. Mean values (±s.d.) across the chronosequence are indicated (*n* = 12).

not fully sun acclimated, which suggests that the cost for producing and maintaining sun leaves was limiting in these ageing trees.

The correlation between the Chl/EPhen and the N/C ratio across the chronosequence was mainly driven by the positive relationship between Chl_m and N_m, which reflects small variations in the amount of nitrogen invested in Chl among sun leaves, independently of stand age. The small size of the subsampling, especially at 95 years, might limit the detection of significant variations in Chl/EPhen and N/C values across the chronosequence. The stability of Chl/EPhen and N/C across the chronosequence suggests (i) no increasing nutrient limitations
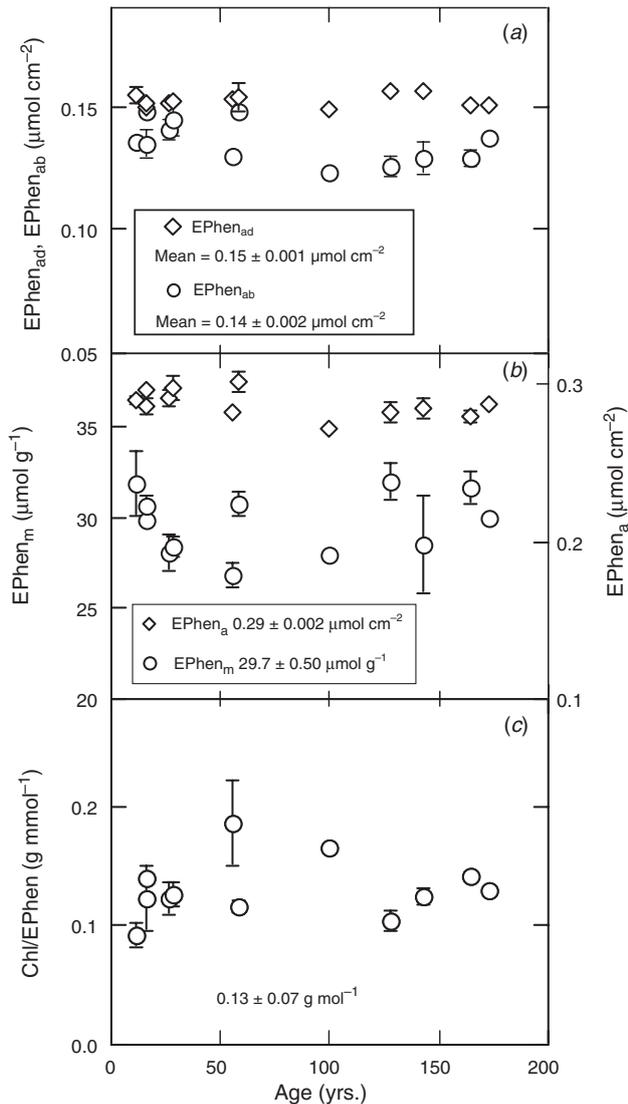
Fig. 6. Variation of area-based adaxial and abaxial (*a*) EPhen content (EPhen_{ad} and EPhen_{ab} respectively), (*b*) area-based EPhen content (EPhen_a) and mass-based EPhen content (EPhen_m) and (*c*) the Chl over EPhen ratio (Chl/EPhen) throughout the chronosequence in beech stands in Exp2. (*b*) EPhen_a obtained from the sum of EPhen_{ad} and EPhen_{ab}; EPhen_m obtained after dividing EPhen_a by LMA. One point corresponds to one stand (1–6 subsampled trees per stand). Error bars represent s.e. Mean values (±s.d.) across the chronosequence are indicated (*n* = 12).

Table 1. Correlation coefficient and significance of the relationships between area-based Chl content (Chl_a) and area-based nitrogen content (N_a), mass-based Chl content (Chl_m) and mass-based nitrogen content (N_m), area-based EPhen content (EPhen_a) and leaf mass per area (LMA), EPhen_a and N_m, mass-based EPhen content (EPhen_m) and N_m, the Chl over EPhen ratio (Chl/EPhen) and N_m, and Chl/EPhen and N/C in Exp2
Note: N/C was chosen rather than C/N to more directly compare to the Chl/EPhen ratio: *n* = 12 stands (1–6 subsampled trees per stand). *, $P < 0.05$; **, $P < 0.01$; n.s., non-significant

Relationship	Correlation coefficient
Chl_a vs N_a	0.746**
Chl_m vs N_m	0.597*
EPhen_a vs LMA	0.329 n.s.
EPhen_a vs N_m	−0.529 n.s
EPhen_m vs N_m	−0.569 n.s
Chl/EPhen vs N_m	0.688*
Chl/EPhen vs N/C	0.659*

pendula, to test whether sun leaves respond to hydraulic constraints throughout a chronosequence.

Conclusion

Optical measurements of EPhen are a useful tool to assess the light microclimate within the canopy and to make leaf sampling from trees more uniform by checking leaf acclimation to light in the field. Stand age-related effects consisted of a proportional microphylly in old stands and a stable sun leaf chemical composition along the chronosequence. Therefore, there was no specific optical signature in leaves indicative of stand age. It was concluded that, along the chronosequence, beech trees invested a constant proportion of mass in N, C, Chl and EPhen per sun leaf, together with reducing leaf size. Therefore, a part of the carbon storage within trees is constantly invested in sun foliage production in ageing stands. Further experiments based on a larger sampling of sun leaves are required to investigate whether old trees actually produced a lower amount of sun leaves in order to estimate how sun leaf production competes with other sinks as carbon availability decreases in ageing trees (Ryan *et al*. 2006). Finally, the stability of sun leaf composition might be related to the unchanged short life span of these deciduous leaves throughout the chronosequence in uniform climatic and edaphic conditions. The pattern may be different in woody perennials, in which leaves are a storage compartment that could be affected by tree ageing (Bond 2000).

Acknowledgements

with ageing, in agreement with Drake *et al*. (2010), (ii) no drought stress due to hydraulic constraints in the upper crown of the oldest trees, in contrast to numerous studies (Ryan *et al*. 2006; Drake *et al*. 2010) and (iii) the stability of sun leaf photosynthetic function. Conversely, it could suggest a lack of plasticity of beech sun leaves, whose characteristic LMA and EPhen saturate with light and therefore might not vary with increasing hydraulic constraints (Niinemets and Kull 1998). Beech is a shade-tolerant species; hence, its sun leaf characteristics might not be strongly affected by tree ontogenesis (Niinemets 2006). Further experiments should be done, especially on plastic shade-intolerant species, like *Betula*

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