CSIRO PUBLISHING
www.publish.csiro.au/journals/fpb *Functional Plant Biology*, 2009, **36**, 732–741

Seasonal changes in optically assessed epidermal phenolic compounds and chlorophyll contents in leaves of sessile oak (*Quercus petraea*): towards signatures of phenological stage

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Abstract. Seasonal patterns of dry mass invested in chlorophyll and epidermal phenolic compounds (EPhen) were investigated *in vivo* using optical methods, in leaves of 2-year-old oaks (*Quercus petraea* Matt. (Liebl.)) grown under semi-controlled conditions. The plasticity of the seasonal pattern was investigated by applying stem girdling treatment. In control young expanding leaves, leaf dry mass per area, dry mass investment in chlorophyll and abaxial EPhen content increased. In late May, at leaf maturity, these variables reached a plateau, and adaxial and abaxial EPhen contents became similar. Thereafter, as leaves aged, dry mass investment in chlorophyll gradually decreased, whereas it remained steady for EPhen. Girdling treatment impacted this seasonal pattern differently depending on the phenological stage. Treatment effects and their reversion revealed *in vivo* EPhen turnover. Finally, optical signatures of immature and mature leaf phenological stages with contrasting nitrogen and carbon economy were proposed, based on the relationship between the chlorophyll to EPhen ratio and the leaf nitrogen to carbon ratio.

Additional keywords: Dualex, girdling, leaf age, LMA, nitrogen, polyphenols, SPAD, UV absorption.

Introduction

Due to the effect of global climate change on ecosystems, there is an increasing need to investigate key parameters of the phenology of deciduous trees in temperate zones. Tree phenology is tightly correlated with leaf structure, phytochemistry and function. These parameters are conventionally described by leaf mass per area (LMA) (Wright *et al*. 2004), allocation of carbon to phenolic compounds (Herms and Mattson 1992) and carbon and nitrogen economy within leaves (Wright *et al*. 2004), respectively. The development of a proximal-sensing method is required to routinely, easily and non-destructively assess these phenological parameters in the field on the same leaves throughout a growth season. Methods based on the optical properties of chlorophyll within a leaf, such as reflectance and transmittance (le Maire *et al*. 2004) and fluorescence (Corp *et al*. 2003; Cartelat *et al*. 2005) can provide non-destructive indicators of leaf structure and function.

Carbon and nitrogen economy within the leaf include the dry mass investment in proteins and phenolic compound synthesis. Proteins are nitrogen-based molecules involved in growth, photosynthesis and homeostasis (Jones and Hartley 1999). In the leaf, 80% of the nitrogen is invested in photosynthetic proteins (Evans 1989), a fraction of which (0.5–1.5%, Niinemets *et al*. 2004) is bound to chlorophyll. Phenolic compounds are carbon-rich molecules including flavonoids, hydroxycinnamic acids, tannins and lignins. They are involved in structure, defence and protection (Jones and Hartley 1999; Wink 1999). The synthesis of protein and that of phenolic compounds, except for a fraction of hydrolysable tannins, compete for the use of a shared precursor, the amino acid L-phenylalanine (PHE) that is assumed to be limiting (Jones and Hartley 1999). The allocation of PHE depends on the stage of leaf development (cf. Jones and Hartley 1999; and the references therein). During leaf initiation, the amount of PHE allocated to phenolic compounds is high for photoprotection at leaf emergence, whereas the amount allocated to proteins is low. In contrast, during leaf expansion, the amount of PHE allocated to phenolic compounds decreases, whereas the amount allocated to proteins increases as photosynthetic protein demand increases (Niinemets *et al*. 2004). At maturity, the amount of PHE allocation to phenolic compounds remains stable and high for photoprotection and stiffening

 10.1071/FP09010 1445-4408/09/080732

(Schultz *et al*. 1982; Jones and Hartley 1999; Salminen *et al.* 2004), whereas the amount allocated to proteins increases for homeostasis. The variations of the protein/phenolic compounds ratio are therefore a potential indicator of the leaf developmental stage. This ratio may provide a useful phenological parameter in the field.

Presently, there is no available proximal-sensing method for assessment of the total protein and phenolic compound content in leaf. However, recently, an optical method assessing *in vivo* contents in chlorophyll and epidermal phenolic compounds (EPhen) has been found to provide signatures of the dry mass invested in protein and phenolic compounds within fully expanded leaves (Meyer *et al*. 2006; Demotes-Mainard *et al*. 2008). These signatures have to be tested throughout the leaf growth season, mainly because the seasonal variation of EPhen content is unknown.

Numerous abiotic (drought, temperature, light, etc) and biotic (herbivores, pathogens) stresses may occur during a growth season and affect the leaf structure, phytochemistry and function, depending on the plasticity of the species. The application of artificial stresses impacting growth and allocation, like stem girdling (Arnold *et al*. 2004; Urban and Alphonsout 2007), elucidates the developmental control of dry mass investment in protein and phenolic compounds. By affecting the sink–source relationship within the tree, stem girdling decreases the availability of carbohydrate for the synthesis of phenolic compounds in expanding leaves that are sinks (Arnold *et al*. 2004). In addition, girdling inhibits photosynthesis, increases carbohydrate accumulation and decreases the leaf nitrogen content in fully expanded leaves (Urban and Alphonsout 2007). In response to carbohydrate accumulation, photosynthetic protein content decreases (Paul and Pellny 2003), whereas phenolic compound content increases (Herms and Mattson 1992). Alteration of the sink–source relationship during the growth season could provide evidence of the potential lability and turnover of phenolic compounds that exist throughout the growth season (Barz and Köster 1981). Kleiner *et al*.'s (1999) pulse-labelling experiment demonstrated a turnover of soluble glycoside phenolic compounds in sink leaves of aspen (*Populus tremuloides* L.). The *in vivo* fluorescence measurements of Bidel *et al*. (2007) have indirectly suggested the lability of phenolic compounds in fully expanded leaves of woody species. However, no experiment has demonstrated the lability of phenolic compounds *in vivo* throughout a growth season.

The objectives of this study were to investigate *in vivo* (i) the seasonal patterns of chlorophyll and EPhen in oak (*Quercus petraea* Matt. (Liebl.)) leaves, (ii) the potential lability of EPhen throughout the growth season using stem girdling treatments and then reversion, and (iii) non-destructive indicators of phenological stages based on the ratio between the dry mass investment in chlorophyll and EPhen.

Materials and methods

Site and plant material

The experiment was conducted in a temperate climate from April 2006 to October 2006, on sessile oaks (*Quercus petraea* Matt. (Liebl.)) obtained from commercial nurseries. They originated from acorns that were sown and grown outdoors in Alençon (48°25′50"N, 00°05′35"E). In December 2005, the 2-year-old trees were planted in an open field on the campus of the University of Paris-Sud (48°42′N, 02°10′E, France, at an elevation of 65 m) in 90 L pots containing a sand/compost mixture (50/50, v/v) without the addition of fertiliser. Trees were spaced 1 m apart. The aboveground portion of the tree was ~1.5 m high. The average temperature and annual rainfall were 11.4°C and 849 mm, respectively (Fig. 1). Compared with previous years, the climate was rainy and hot during the summer, with a 3-week heat wave in July (DOY 191–210, Fig. 1). Budburst took place between day of year (DOY) 100 (mid-April) and DOY 135 (mid-May). The plantation was watered once a week (12 L of water per pot). Caterpillars were removed by hand, and diseases were controlled by two pesticide applications (DOY 173 and DOY 194) against aphids, mealy bugs and powdery mildew (Decis, deltamethrin; Vertimec, abamectin; Systhane, myclobutanil; Puteaux SA, Les Clayes ss Bois, France). For all treatments, the pots were wrapped with plastic bags sealed around the trunks to stop the rainfall water supply. The experiment was carried out only on the first leaf flush. A second flush occurred in June.

Experimental design

The trees were cultivated under two treatments arranged in a completely randomised design. Twenty five trees were cultivated in control conditions and 21 trees were stem-girdled (G). Optical and biochemical measurements were performed weekly from 27 April (DOY 117) to 10 October (DOY 283), on the leaves that expanded out between DOY 117 and DOY 130. Leaf fall occurred in October. Leaf chlorophyll and EPhen were optically assessed *in situ* using 20–25 south-facing leaves per treatment and per date. Three to seven optical measurements per leaf were performed

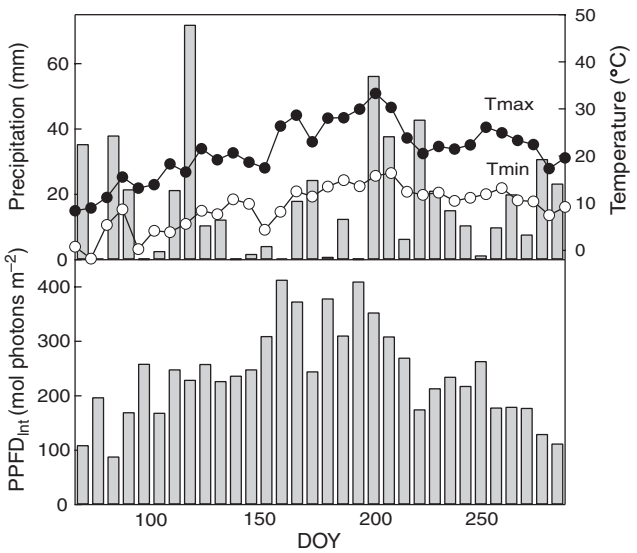

Fig. 1. Weekly means of minimum (Tmin, open circles) and maximum (Tmax, closed circles) temperatures, weekly sums of precipitation and weekly integrated PPFD (PPFD$_{int}$) from DOY 65 (March) to DOY 290 (October) in 2006. Annual precipitation in 2006 was 849 mm and the mean temperature was 11.4°C.

depending on the size of the leaf. Dualex measurements were performed on both sides of the leaves. In addition, three leaves were harvested per treatment and per week at around 1100 hours for optical, LMA and biochemical (chlorophyll, nitrogen, carbon and phenolic compound) measurements. The LMA used for mass-based content calculation is the average obtained from the leaves sampled every week.

Girdling

Girdling was applied three times on three different sets of seven trees. The first girdling (GA) was applied just after bud-burst on DOY 129; the second girdling (GB) on DOY 181, during trunk growth when leaves were mature; the third girdling (GC) on DOY 244, just after trunk growth stopped at the beginning of leaf senescence. Girdling was achieved by removing 2 cm of bark and phloem all around the trunk, 30 cm above the collar. The girdled zone was then protected using parafilm. The girdling periods lasted 41 and 67 days for GA and GB treatments, respectively. After these periods, trunk phloem tissues were regenerated, and the phloem sap-flow restarted, for GA and GB trees. No tissue regeneration occurred after the GC girdling that lasted until fall.

In vivo *optical estimation of leaf chlorophyll and EPhen contents*

Chlorophyll and EPhen were measured in the field using two portable leaf-clip meters. Chlorophyll content was estimated by the sequential measurement of transmission through leaves of red (650 nm) and infrared (940 nm) light, using a portable Minolta SPAD-502 chlorophyll meter (Konica-Minolta, Carrière-sur-Seine, France; hereafter referred to as SPAD). The difference in transmission at these two wavelengths was an indicator of chlorophyll content per unit leaf area (Markwell *et al*. 1995). A calibration curve was established using chlorophyll measurements of leaf extracts, and the SPAD values were converted into area-based chlorophyll content (Chl_a). The mass-based chlorophyll content, Chl_m, was obtained by dividing Chl_a by LMA ($Chl_a = Chl_m \times LMA$).

EPhen were assessed by the sequential measurement of leaf chlorophyll fluorescence excited by UV (375 nm) and red (650 nm) light, using a FORCE-A Dualex meter (Force-A, Orsay, France; hereafter referred to as Dualex) (Goulas *et al*. 2004) according to Cartelat *et al*. (2005). EPhen content was deduced, based on the UV-absorbing properties of the leaf, as first described by Bilger *et al*. (1997, 2001). EPhen absorbed UV radiation only and are mainly water-soluble glycosylated flavonoids stored in vacuoles and hydroxycinnamic acids bound to cell walls. In oaks, the main absorbers detected at 375 nm are flavonols. Red light was used as a reference, as it crossed the epidermis without being absorbed to reach the chlorophyll in the mesophyll. The ratio of chlorophyll fluorescence emission (from 695 nm) under excitation by UV and red light was an index of EPhen content per unit leaf area. 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 ($EPhen_a = EPhen_m \times LMA$). $EPhen_a$ is expressed in molar units of quercetin 3-O-glucoside (quercitrin) equivalents, calculated from Dualex-derived absorbance according to Meyer *et al*. (2006), using the molar extinction coefficient (ε) of 9.7 $\mu mol^{-1}\,cm^2$ (9.7 $mM^{-1}\,cm^{-1}$) at 375 nm according to Cerovic *et al*. (2008).

The area-based values of chlorophyll and epidermal phenolic compounds incorporate two variables: (i) the proportion of the leaf dry mass invested in chlorophyll and EPhen that depends on the competition between protein and phenolic compound synthesis, and (ii) the dry mass accumulation per unit leaf area (LMA) in which chlorophyll and EPhen are diluted (Meyer *et al*. 2006). The ratio between chlorophyll and EPhen content (Chl/EPhen ratio) assessed on the same leaf provides a signature of dry mass invested in protein and phenolic compounds at a given time (Cartelat *et al*. 2005; Demotes-Mainard *et al*. 2008).

Leaf chlorophyll and phenolic compound extraction and leaf mass per area

Leaf area was measured using an area meter (Delta-T Devices, UK). Four or six leaf discs (0.55 mm diameter), corresponding to SPAD and Dualex measurements, were sampled. Leaves and discs were frozen in liquid nitrogen and stored at −80°C before freeze-drying. Leaves were then weighed in order to calculate the LMA. Extraction of chlorophyll from two or three discs was performed in 100% methanol at 60°C for 2 h in a sealed tube. After cooling, the absorption of the extract was measured with a spectrophotometer (Hewlett Packard, Les Ulis, France). Chlorophyll *a* and *b* were calculated according to Porra *et al*. (1989). Absorption at 280, 320 and 375 nm after subtraction of chlorophyll contribution was used to estimate levels of soluble phenolic compounds (Cerovic *et al*. 2002).

Nitrogen and carbon content

Leaf discs were weighed and encapsulated into tin-capsules (Courtage Analyse Service, Mont Saint-Aignan, France) for nitrogen analyses. One milligram of the remaining leaves was weighed and encapsulated into tin-capsules for carbon analysis. The samples were analysed for C and N percentages at the Paris-XI University with an NA–1500 elemental analyser (Carlo Erba, Milan, Italy).

Statistical analysis

Statistical analysis was carried out with Statistica 6.1 (StatSoft Inc., Maison-Alfort, France) and Igor PRO 6.03 (WaveMetrics Inc., Lake Oswego, OR, USA). The effects of DOY, treatments and their interaction during the defined treatment periods were tested using repeated-measures analysis of variance (ANOVA) according to the GLM procedure. The analysis was performed on mean values per tree. In cases where normality of the residuals and homoscedasticity were violated, the data were log-transformed. The relationships between absorbance of extracts and Dualex measurements, between Chl_m and N_m and between Chl/EPhen and N/C, were investigated using correlation and regression procedures. For each date and treatment, the sampling size was $n = 3$ and $n = 20$–25 replicates for

biochemical and optical measurements, respectively. In Figs 2 and 3, error bars are 95% confidence intervals.

Results

Seasonal patterns in control and treated trees

In control, all considered variables varied widely with season (Figs 2, 3), except leaf mass-based carbon content (C_m), which remained constant at around 46% (Fig. 2*b*). In control trees, there was an increase in Chl_a from DOY 117 to DOY 150 (Fig. 2*c*) that was mainly ascribed to an increase of LMA as leaves grew (Fig. 2*a*). This increase contrasted with the decrease in N_m during this period (Fig. 2*b*). Thereafter, from DOY 150 onwards, Chl_a remained stable for one month, and then gradually decreased (Fig. 2*c*). This was mainly ascribed to the variation of Chl_m (Fig. 2*d*) since LMA remained more or less stable during this period (Fig. 2*a*). Leaf expansion ceased at DOY 170 (not shown). From DOY 150 onward, the variation of N_m was parallel to that of Chl_m (Fig. 2*b*, *d*). The seasonal variation of $EPhen_a$ was slight and ascribed mainly to that of LMA (Figs 2*a*, 3*a*). $EPhen_m$ decreased slightly during leaf expansion and then remained stable from DOY 130 onward (Fig. 3*b*). The Chl/EPhen ratio increased rapidly during the period of leaf growth from DOY 117 to DOY 150 and then remained more or less stable for one month and finally decreased gradually from DOY 170 to fall (Fig. 3*c*). Leaves emerged with low $EPhen_{ab}$ and high $EPhen_{ad}$ (Fig. 3*d*). The increase of $EPhen_{ab}$ from DOY 130 to DOY 170 induced the increase of $EPhen_a$. From DOY 170 onwards, $EPhen_{ab}$ and $EPhen_{ad}$ were similar (Fig. 3*d–f*) and LMA was constant; thus, $EPhen_a$ remained stable.

Only girdling treatments GB and GC significantly increased LMA (Fig. 2*a*, Table 1). The effect of GA treatment was not immediate and occurred 2 weeks after the beginning of the treatment, when leaves were reaching maturity. GA treatment did not affect the final leaf size (not shown). Only GB treatment slightly, but significantly, increased C_m ($P = 0.049$) and decreased N_m ($P = 0.041$, Fig. 2*b*). This was related to the increase in LMA that resulted from an increase in tissue density and/or thickness. GA treatment significantly attenuated the rise of Chl_a during the period of leaf growth (Fig. 2*c*, Table 1). This effect was ascribed to the strong and significant decrease in Chl_m that largely compensated for the increase of LMA (Fig. 2*d*, Table 1). At leaf maturity (DOY 156), Chl_m was 27% lower than the control value; this effect was reversible. GB and GC treatments did not affect Chl_a, since the increase in LMA compensated for the decrease in Chl_m, although this latter was not significant in GB (Table 1). Only GB and GC treatments significantly decreased $EPhen_a$ due to a decrease in both $EPhen_{ad}$ and $EPhen_{ab}$, whereas GA treatment specifically

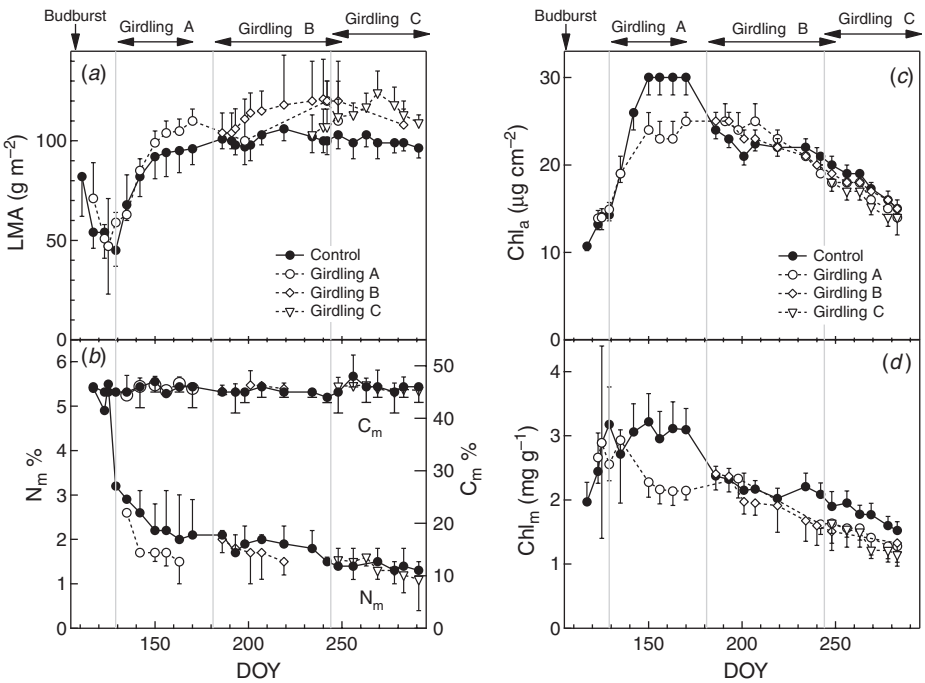

Fig. 2. Comparison of seasonal variations in control and girdled oak trees of (*a*) leaf mass per area, LMA, (*b*) mass-based leaf nitrogen (N_m) and carbon contents (C_m), (*c*) area-based chlorophyll content (Chl_a) and (*d*) mass-based chlorophyll content (Chl_m) obtained by dividing Chl_a by LMA (see Materials and methods). (*c*) area-based data obtained from optical measurements; (*d*) mass-based data obtained after dividing optical measurement by LMA. From left to right, the vertical lines indicate the beginning of the GA, GB and GC girdling treatments, respectively, and the horizontal top arrows indicate the periods of GA, GB and GC girdling treatments, respectively. Error bars are 95% confidence intervals ($n = 3$ for C_m and N_m, $n = 8$ for LMA, $n = 25$ for Chl_a and Chl_m). Control (filled circles), GA (open circles), GB (open diamonds) and GC (inverted open triangles).

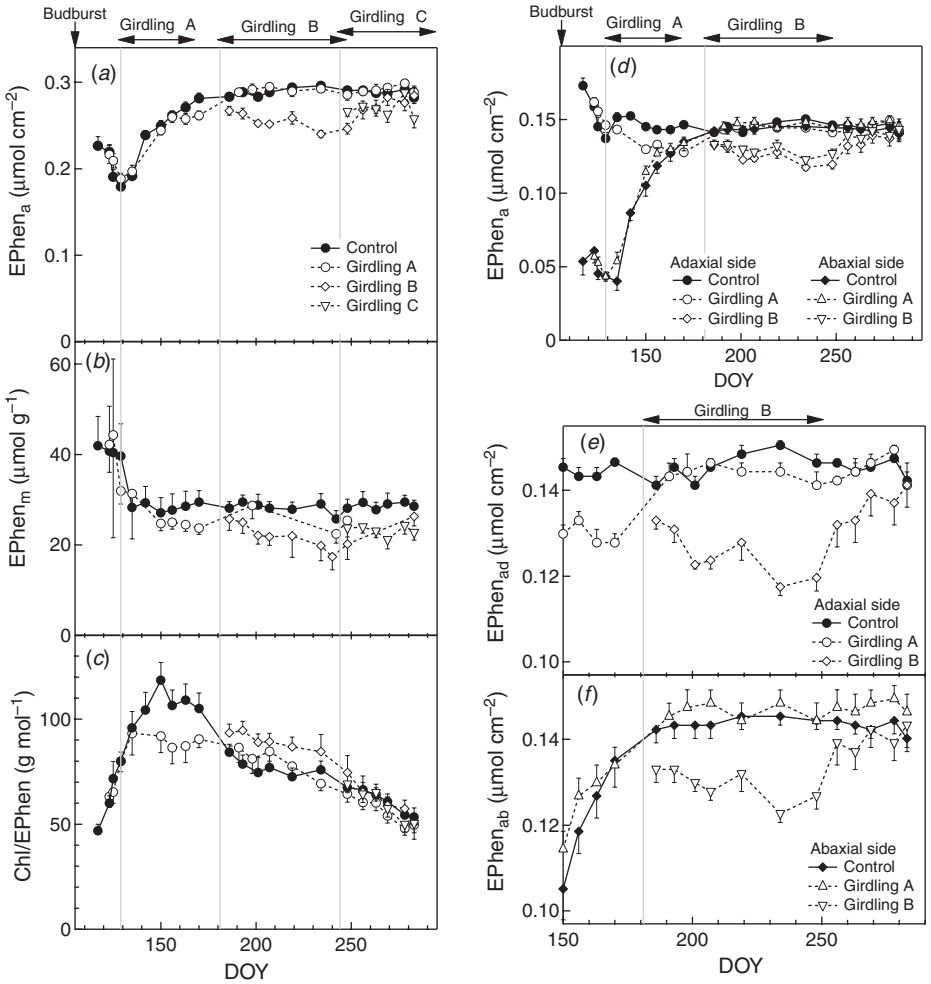

Fig. 3. Comparison of seasonal variations in control and girdled oak trees of (*a*) area-based epidermal phenolic compound content (EPhen$_a$), (*b*) mass-based EPhen content (EPhen$_m$) obtained by dividing EPhen$_a$ by LMA (see Materials and methods), (*c*) chlorophyll over EPhen ratio (Chl/EPhen), (*d*) area-based adaxial and abaxial EPhen content (EPhen$_{ad}$ and EPhen$_{ab}$). (*e*, *f*), zooms of the panel (*d*) showing the variations of EPhen$_{ad}$ (*e*) and EPhen$_{ab}$ (*f*) from DOY 151onwards. In (*a–c*), the lines, the arrows and the symbols are as in Fig. 2. In (*d*), from left to right, the vertical lines indicate the beginning of the GA and GB girdling treatments, respectively, and the horizontal top arrows indicate the periods of the GA and GB girdling treatments, respectively. In (*e*) and (*f*), the vertical line indicates the beginning of the GB girdling treatment and the horizontal top arrow indicates the period of the GB treatment. Error bars are 95% confidence intervals ($n = 25$). In (*d–f*), data from control, GA and GB girdling treatments are drawn as filled circles, open circle and open diamonds, respectively, for adaxial leaf side, and closed diamonds, open triangles and inverted open triangles, respectively, for abaxial leaf side.

decreased EPhen$_{ad}$ (Fig. 3*d–f*, Table 1). The effects of GA and GB treatments on EPhen content were reversible. GC trees never recovered from the treatment, so there was no reversion of EPhen content to control values. Figure 3*c* and Table 1 show that GA treatment induced a significant and reversible decrease of 20–30% in the Chl/EPhen ratio as leaves stopped growing (DOY 140–170). This resulted from a decrease in the investment of dry mass in chlorophyll. GB and GC treatments did not significantly affect the Chl/EPhen ratio, although Fig. 3*c* shows an increasing trend in response to GB treatment resulting from the significant decrease in EPhen$_m$. GC treatment did not affect the Chl/EPhen ratio since chlorophyll and EPhen were similarly reduced.

Assessment of phenolic compounds by UV absorption

In leaves from control trees, the seasonal pattern of UV absorption by total soluble phenolic compounds was close to that of EPhen$_a$ for every considered wavelength (Figs 3*a*, 4). Throughout the period of rapid EPhen$_a$ changes (DOY 117–170), control Dualex measurements were positively and tightly correlated with the extract UV absorption

Table 1. Repeated-measures ANOVA for effects of DOY, treatments and the DOY × Treatment (Treat.) interaction on leaf mass per area (LMA, $g\,m^{-2}$), area-based chlorophyll content (Chl_a, $\mu g\,cm^{-2}$), mass-based chlorophyll content (Chl_m, $mg\,g^{-1}$), area-based epidermal phenolic compound content ($EPhen_a$, $\mu mol\,cm^{-2}$), area-based adaxial EPhen content ($EPhen_{ad}$, $\mu mol\,cm^{-2}$), area-based abaxial EPhen content ($EPhen_{ab}$, $\mu mol\,cm^{-2}$), mass-based EPhen content ($EPhen_m$, $\mu mol\,g^{-1}$) and the chlorophyll to EPhen ratio (Chl/EPhen, $g\,mol^{-1}$)

F-values, d.f. (numerator, denominator) and significance of the results from ANOVA are indicated. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. d.f., degrees of freedom; n.s., non-significant; *n*, total sample size; *p*, number of groups

		Girdling (GA) effect (DOY 132–171)		Girdling (GB) effect (DOY 185–243)		Girdling (GC) effect (DOY 244–291)	
		d.f. [*p*–1, *n*–*p*]	*F*-value	d.f. [*p*–1, *n*–*p*]	*F*-value	d.f. [*p*–1, *n*–*p*]	*F*-value
LMA	DOY	[5, 60]	35.9***	[7, 80]	1.3 n.s.	[8, 94]	1.2 n.s.
	Treatment	[1, 63]	2.7 n.s.	[1, 86]	12.1*	[1, 101]	10.5*
	DOY × Treat.	[5, 60]	1.2 n.s.	[7, 80]	2.1 n.s.	[8, 94]	0.6 n.s.
Chl_a	DOY	[4, 51]	35.7***	[6, 63]	7.8***	[5, 68]	13.2***
	Treatment	[1, 54]	8.9*	[1, 68]	0.0 n.s.	[1, 70]	2.4 n.s.
	DOY × Treat.	[4, 51]	6.4***	[6, 63]	2.1 n.s.	[5, 68]	0.5 n.s.
Chl_m	DOY	[4, 51]	0.4 n.s.	[6, 63]	15.5***	[5, 68]	23.1***
	Treatment	[1, 54]	15.2**	[1, 68]	2.0 n.s.	[1, 70]	12.9**
	DOY × Treat.	[4, 51]	3.7*	[6, 63]	4.9***	[5, 68]	1.2 n.s.
$EPhen_a$	DOY	[4, 51]	217.3***	[6, 63]	62.2***	[5, 68]	5.5***
	Treatment	[1, 54]	0.8 n.s.	[1, 68]	59.6***	[1, 70]	40.0***
	DOY × Treat.	[4, 51]	3.6*	[6, 63]	7.5***	[5, 68]	1.7 n.s.
$EPhen_{ad}$	DOY	[4, 51]	8.0***	[6, 63]	41.9***	[5, 68]	7.2***
	Treatment	[1, 54]	15.6**	[1, 68]	104.0***	[1, 70]	16.7**
	DOY × Treat.	[4, 51]	11.8***	[6, 63]	8.3***	[5, 68]	2.2 n.s.
$EPhen_{ab}$	DOY	[4, 51]	354.6***	[6, 63]	44.5***	[5, 68]	2.7*
	Treatment	[1, 54]	0.1 n.s.	[1, 68]	24.7**	[1, 70]	13.1**
	DOY × Treat.	[4, 51]	1.1 n.s.	[6, 63]	2.6*	[5, 68]	1.1 n.s.
$EPhen_m$	DOY	[4, 51]	59.7***	[6, 61]	109.0***	[5, 68]	7.4***
	Treatment	[1, 54]	3.5 n.s.	[1, 68]	190.9***	[1, 70]	407.4***
	DOY × Treat.	[4, 51]	6.1**	[6, 61]	32.5***	[5, 68]	5.5***
Chl/EPhen	DOY	[4, 51]	8.3***	[6, 63]	2.9*	[5, 68]	14.7***
	Treatment	[1, 54]	9.2*	[1, 68]	3.1 n.s.	[1, 70]	0.1 n.s.
	DOY × Treat.	[4, 51]	2.6 n.s.	[6, 63]	0.5 n.s.	[5, 68]	0.4 n.s.

(Fig. 4 insets). The correlation was higher when UV was set at 375 nm ($r^2=0.90$) than at 320 nm ($r^2=0.87$) and at 280 nm ($r^2=0.45$). This was expected since Dualex mainly measured flavonols, the main absorption peak for which is ~370 nm (Cerovic *et al*. 2002). These correlations indicate that Dualex measurement could be used as an index of total soluble phenolic compounds in oak leaf.

For girdled trees, leaf total soluble phenolic compound absorption was higher than what could be expected from Fig. 3*a*. For GA treatment, the difference between total soluble phenolic compounds and $EPhen_a$ was the highest at 280 nm, with an increase in absorbing compounds in response to treatment. These compounds could be catechins, sugars and lipids. The tail of their absorption spectra could be responsible for the enhancement of the total soluble Phen absorption at 375 nm and 320 nm (Fig. 4*a*, *b*). Preliminary HPLC analysis of leaf phenolic compounds suggested that catechin oligomers increased in response to GA and GB treatments (not shown). However, since $EPhen_a$ did not increase in response to these treatments (Fig. 3*a*), these additional soluble phenolic compounds should be located in the mesophyll. Since Dualex cannot detect the accumulation of phenolic compounds in the mesophyll, Dualex measurements are not a surrogate for total leaf soluble phenolic compounds in girdled trees, which is an artificial situation. However, Dualex could be used coupled with extraction to distinguish between epidermal and mesophyll phenolic compounds.

Relationship between optically assessed Chl and EPhen and leaf carbon and nitrogen content

The Chl_m *v.* N_m relationship varied according to leaf age in control and girdled trees (Fig. 5). Directly after bud-burst, from DOY 117 to DOY 125, N_m and Chl_m were high and low, respectively. From DOY 129 to DOY 150, Chl_m was more or less constant; N_m decreased due to a dilution effect by dry mass accumulation. Hence, from bud-burst to DOY 150, Chl_m was not correlated with N_m and varied negatively with N_m. From DOY 150 to DOY 283, both Chl_m and N_m gradually decreased; therefore, Chl_m was positively correlated with N_m ($r^2=0.68$). Hence, Chl_m can be regarded as an index for total leaf protein only in mature leaves from DOY 150 onwards.

The relationship between the Chl/EPhen ratio and the leaf nitrogen to carbon ratio (N/C) (Fig. 6) demonstrates a drastic change in carbon and nitrogen economy between young expanding and mature leaves in control trees. The correlation between these ratios was negative and tight ($r^2=0.83$) in expanding leaves and positive and tight ($r^2=0.67$) in mature

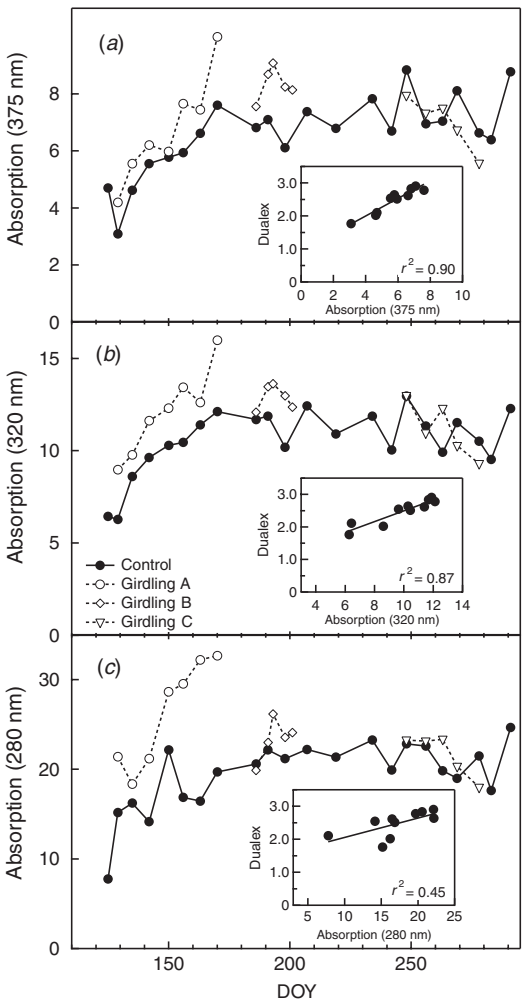

Fig. 4. Comparison of seasonal variations in control and girdled trees of the absorption of the leaves methanolic extracts at (*a*) 375 nm, (*b*) 320 nm and (*c*) 280 nm. The GA, GB and GC girdling treatments were applied on DOY 129, DOY 181 and DOY 244, respectively. The symbols are as in Fig. 2. The inserts show the relationship between Dualex measurements and leaves extract absorption in control trees from DOY 117 to DOY 170. Regression equations are $\text{Dualex} = 0.93 + 0.27 \times A_{375}$, $n = 10$, $r^2 = 0.90$; $\text{Dualex} = 0.84 + 0.16 \times A_{320}$, $n = 10$, $r^2 = 0.87$; $\text{Dualex} = 1.47 + 0.06 \times A_{280}$, $n = 10$, $r^2 = 0.45$, for (*a*), (*b*) and (*c*), respectively.

and aging leaves. The variations in Chl_m and N_m drove these relationships (see Fig. 2*b*, *d*).

Discussion

Seasonal patterns of optically assessed chlorophyll and EPhen

The seasonal patterns of LMA, chlorophyll and EPhen contents revealed the changes of leaf structure and function throughout the growth season. Especially, the seasonal pattern of EPhen content revealed leaves photoprotection against natural UV light during the growth season since EPhen synthesis is mainly driven by UV-B light (Krizek *et al*. 1993; Bidel *et al*. 2007). Based on the seasonal changes of all parameters, four phenological periods could be distinguished.

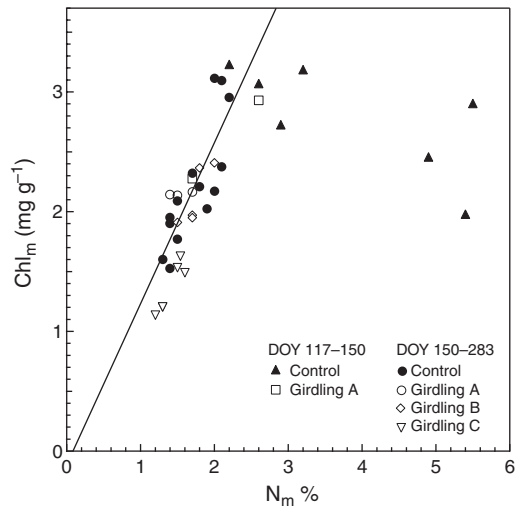

Fig. 5. Relationship between the optically assessed mass-based chlorophyll content (Chl_m) and the mass-based nitrogen content (N_m) of oak leaves for control and girdled trees. The GA, GB and GC girdling treatments were applied on DOY 129, DOY 181 and DOY 244, respectively. Regression equation for control is $Chl_m = -0.11 + 1.35 \times N_m$, $n = 14$, $r^2 = 0.68$. From DOY 117 to DOY 150, data from control and GA girdling treatment are drawn as filled triangles and open squares, respectively. From DOY 150 to DOY 283, control (filled circles), GA (open circles), GB (open diamonds) and GC (inverted open triangles) girdling treatments.

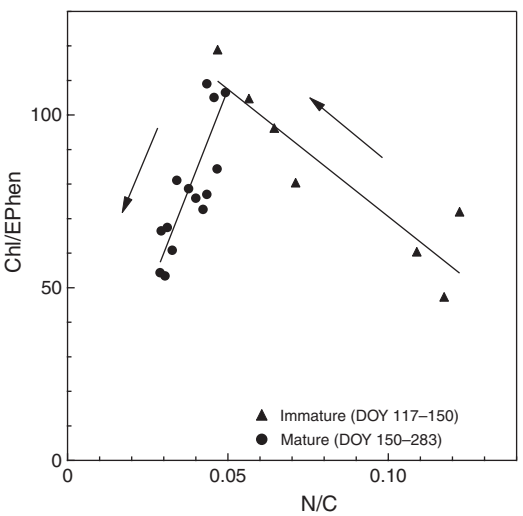

Fig. 6. Relationship between the chlorophyll to epidermal phenolic compound content ratio (Chl/EPhen) and the nitrogen to carbon content ratio (N/C) in immature (triangles) and mature (circles) leaves from control trees. Note that N/C was chosen rather than C/N to more directly compare the both ratios. Arrows indicate increasing leaf age. Regression equations are $\text{Chl/EPhen} = 144.3 - 736.8 \times \text{N/C}$, $n = 7$, $r^2 = 0.83$, $\text{Chl/EPhen} = -2.83 + 2117 \times \text{N/C}$, $n = 14$, $r^2 = 0.67$, for DOY 117–150 and DOY 150–283, respectively.

In a first period (from bud-burst to DOY 129, corresponding to early May), at bud-burst, leaves developed a high demand for phenolic compounds for photoprotection in buds and

during unfurling, in accordance with Jones and Hartley (1999) and Salminen *et al*. (2004). EPhen accumulated early on the adaxial side, which is the most exposed to light. The week after the bud-burst (DOY 125), the rise in Chl_m indicated high photosynthetic proteins demand before leaf expansion.

In a second period (from DOY 130 to DOY 150, corresponding to late May), the increase of the Chl/EPhen ratio represents the high investment of dry mass in photosynthetic proteins compared with low demand for phenolic compound synthesis, in accordance with Jones and Hartley (1999). At the beginning of this period, leaves were sinks, importing material from tree reserves (Turgeon 1989). Leaves were both expanding and increasing in mass. The mass increase induces a dilution of $EPhen_m$ and to a lesser extent a dilution of Chl_m, which was actively synthesised during this period, in accordance with Jones and Hartley (1999) and Salminen *et al*. (2004). Furthermore, the accumulation of EPhen in abaxial leaf side resulted from an induction of expression of phenylalanine ammonia lyase (PAL) and chalcone synthase by visible light and UV-B (Krizek *et al*. 1993), demonstrating that leaves were acclimating to light. Therefore, as reported by Hughes *et al*. (2007) for various deciduous species, leaves simultaneously developed photosynthetic competence and full photoprotection.

In a third period (from DOY 151 to DOY 180, corresponding to June), leaf growth stopped. Fully expanded leaves reached maturity and became photosynthetically competent sources that were also photoprotected (Hughes *et al*. 2007). The constancy of $EPhen_m$ was not in accordance with the stiffening of leaves suggested by high LMA and reported by Jones and Hartley (1999). During stiffening, phenolic compounds polymerised into large molecules, like lignin and condensed tannins (Salminen *et al*. 2004) that Dualex cannot measure.

In a fourth period (from DOY 181 to DOY 283, corresponding to July–September), the decrease in chlorophyll content and in the Chl/EPhen ratio suggested that leaves were exporting nitrogen, especially from chlorophyll, in accordance with Niinemets *et al*. (2004). The constancy of $EPhen_m$ was in accordance with Jones and Hartley (1999) and Salminen *et al*. (2004) and suggests that EPhen were either conserved or synthesised and destroyed at the same rate. This suggests that there is a constitutive and genetically programmed level of EPhen independent of climatic variations during summer, and that leaves were steadily protected against UV light during the initial stages of nitrogen remobilisation.

In leaves of *Quercus robur* L., a closely interfertile species of *Quercus petraea* (Matt.) Liebl. (Bacilieri *et al*. 1996), hydrolysable tannins, flavonoid glycosides and proanthocyanidins are the major phenolic groups (Salminen *et al*. 2004). The observed seasonal pattern of EPhen was similar to that reported by Salminen *et al*. (2004), not only for total flavonoid glycosides and hydrolysable tannins, but also for total phenolic compounds, estimated by chemical analysis. This confirms a role for EPhen as an empirical index of total phenolic compounds in leaves (Meyer *et al*. 2006). In addition, optical measurements of EPhen allow researchers to assess the light acclimation of developing leaves; full light acclimation being achieved when $EPhen_{ab}$ is high and similar to $EPhen_{ad}$.

Plasticity of the seasonal pattern and lability of EPhen

The application of stem girdling was a way to analyse the plasticity of leaf development, as well as the lability of leaf components. Girdling effects differed according to phenological stages. In sink leaves, based on the studies by Arnold *et al*. (2004) and Urban and Alphonsout (2007), a decrease in protein demand for photosynthesis and an increase in demand for phenolic compounds are expected in response to girdling. However, we did not observe this trend *in vivo*. It is likely that sink leaves import carbohydrates from stem reserves of the 2-year-old trees, in contrast to the study by Arnold *et al*. (2004), which used saplings. Girdling affects the dry mass investment only from DOY 140–150 onwards, suggesting that the sink-source transition occurred at this DOY, before leaf maturity, in accordance with Turgeon (1989). The second treatment (GB) induced a decrease of leaf photosynthetic capacity by around 85% from DOY 220 to DOY 242, the control value being ~7.9 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$ (Maunoury-Danger 2007). This is interpreted as negative feedback on photosynthesis due to a source–sink imbalance caused by the accumulation of carbohydrate in leaves (Goldschmidt and Huber 1992; Layne and Flore 1995; Iglesias *et al*. 2002). Despite carbohydrate accumulation, $EPhen_m$ decreased unexpectedly in GB-trees, but preliminary HPLC analyses (not shown; cf. Fig. 4) indicated that leaves accumulated catechins, flavonols and condensed tannins in mesophyll. Therefore, girdling might induce a change in location of phenolic compounds, with a decrease in flavonoid accumulation in epidermis and an increase in mesophyll. Girdling did not affect the leaf acclimation to light since the $EPhen_{ab}$ content was similar to that of control leaves in GA-trees. This suggests that light-induced PAL activity drove strong demand for synthesis of phenolic compounds in abaxial epidermis during leaf growth.

Girdling treatments and their reversion showed for the first time the lability of EPhen *in vivo*. $EPhen_m$ of GA and GB-trees decreased compared with control trees and remained unchanged as long as the girdling was maintained (Fig. 3*b*). After reversion, $EPhen_m$ returned to baseline levels. For GC-trees, there was no reversion. The decrease in EPhen to a stable level is in accordance with the presence of two pools of phenolic compounds, a constitutive one and a labile one. Labile phenolic compounds are lost during girdling and synthesised again when the phloem reverses. They may be degraded, polymerised or exported into the mesophyll. This *in vivo* lability of EPhen is in accordance with findings by Kleiner *et al*. (1999) showing the rapid turnover of glycoside phenolic compounds in aspen leaf using ^{14}C labelling.

Optical signatures of phenology

From optical measurements, we identified immature (before DOY 150) and mature (after DOY 150) phenological stages with contrasting nitrogen and carbon economy. In immature leaves, nitrogen was not allocated to leaves at the precise rate at which the photosynthetic apparatus develops, leading to opposing variations in Chl_m and N_m. In mature leaves, the parallel decrease in Chl_m and N_m was related to mid- and late-seasonal re-mobilisation of leaf nitrogen content. These results

are in accordance with Reich *et al*. (1991) and Niinemets *et al*. (2004). In addition, carbon was invested in EPhen throughout the season, with an increase in $EPhen_{ab}$ in immature leaves. The optical signatures of immature leaves could be empirically defined by (i) the negative Chl/EPhen *v.* N/C relationship, which could be assessed in the field by a temporal increase in the ratio between SPAD and Dualex values, and (ii) different $EPhen_{ad}$ and $EPhen_{ab}$ values. Optical signatures of mature leaves could be defined by (i) the positive correlation between Chl/EPhen *v.* N/C ratios, which could be assessed in the field by a temporal decrease in the ratio between SPAD and Dualex values, and (ii) similar $EPhen_{ad}$ and $EPhen_{ab}$ values. These signatures include dilution due to the accumulation of dry mass, the carbon and nitrogen economy of the leaf and the degree to which the leaf epidermis has acclimated to light. It is worth noting that, depending on species and leaf exposition to sunlight, $EPhen_{ab}$ could differ from $EPhen_{ad}$ in fully expanded leaves; however, the two parameters remained tightly and positively correlated (Barthod *et al*. 2007; Demotes-Mainard *et al*. 2008).

In conclusion, further experiments have to be done to validate these signatures in other deciduous species of trees from temperate forest. These signatures, easy to monitor rapidly, could be useful for proximal-sensing tree phenology, in order to probe *in vivo* the maturity state of the leaves and to estimate their role in the carbon production of the entire plant.

Acknowledgements

This work was supported by the CNRS, the company FORCE-A, and the Essonne country through the project ASTRE. We thank Claire Bouchut (laboratory 'Science pour l'oenologie', Montpellier, France) for technical assistance in HPLC analysis. We are grateful to Dr Gérard Lacroix (Laboratoire Bioemco, ENS, France), Dr Michael Danger (Laboratoire Bioemco, ENS, France), Marine Le Moigne (Force-A, Université Paris Sud, France) and Dr Erwin Dreyer (Ecologie et Ecophysiologie forestières, INRA-UHP1137, France) for very helpful comments on the manuscript. American Journal Experts (Durham, USA) reviewed the paper for English usage.

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Manuscript received 10 January 2009, accepted 22 June 2009