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ORIGINAL PAPER

Differences in pigment circadian rhythmicity in green- and red-leafed tree species in the sun and shade

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Abstract Light flux and quality are crucial factor for setting endogenous plant circadian rhythms. Evaluating the daily rhythmicity of leaf chlorophyll content is an effective method to monitor the plant physiological endogenous clock in response to environmental signals such as light availability/quality. Here, we used a leaf-clip sensor to monitor diurnal rhythms in the content of chlorophyll and flavonoids such as flavonols and anthocyanins in three green- (*Ailanthus altissima*, *Tilia platyphyllos* and *Platanus*×*acerifolia*) and two red-leafed (*Acer platanoides* cv. Crimson King and *Prunus cerasifera* var. *pissardii*) tree species, adapted to sun (L) or shade (S). Significant differences in chlorophyll content (Chl) and its variations during the day were observed among treatments in all the analyzed species. S-plants had more Chl than L-plants irrespective of leaf color, and Chl variations were more distinct during the day than in L-plants. In particular, contents were lowest in the morning (9:00) and in the middle of the day (at 12:00 and 15:00), and the highest at dusk (21:00). The less evident trends in Chl variation in L-plants were attributed to a decrease in Chl content in high light, which likely masked any increases in the shaded counterparts during the afternoon. Daily flavonol levels did not vary no notably during the day. In sun-exposed red leaves, anthocyanins partially screened mesophyll cells from incident light, and its levels were similar to the Chl dynamics in the shaded counterparts. This study provides new bases for further work on endogenous rhythms of plant pigments and improves our understanding of plant physiology in the context of day/night rhythmicity.

Keywords Anthocyanins · Chlorophylls · Circadian clock · Flavonols · Shade syndrome

Introduction

The circadian clock is an endogenous 24-h timer that plays a fundamental role, synchronising physiological, biochemical and developmental processes in plants with the outside world (Barak et al. 2000; Inoue et al. 2018; Lee et al. 2021). Light/dark or temperature fluctuations act as time-keeping signals to a plant oscillator, resetting or extending a biological plant phase (Barak et al. 2000; Soengas et al. 2018). Synchrony of the plant endogenous rhythms to environmental rhythms (e.g., light and temperature) confers photosynthetic advantages, improving plant growth and performances and promoting plant acclimation to external stimuli (Dodd et al. 2005). Light quantity and quality are important factors to mark the endogenous circadian rhythms in plants. The light signal operates primarily by modulating the transcription of numerous genes related to plant physiological processes (Rugnone et al. 2013). In molecular studies of herbaceous species as *Arabidopsis thaliana*, the core oscillator of the circadian clock was found to comprise evening- and morning-phased transcription factors (Wang and Tobin 1998; Barak et al. 2000; Rugnone et al. 2013; Nakamichi 2020). Furthermore, many of the key genes related to the

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biosynthesis of light-harvesting complexes, pigments, and pigment-binding proteins fluctuate synchronously with the day/night cycle (Harmer et al. 2000; Covington et al. 2008; Schmid 2008; Khan et al. 2010).

The synthesis/degradation cycle of chlorophyll and of non-covalently bound light-harvesting proteins (Schmid 2008) are markers of the chlorophyll daily rhythmicity in leaves; thus, analysis of the daily rhythmicity of chlorophyll content in leaves is a rapid method to characterize the endogenous clock in plants in response to daily environmental variations (Samsone et al. 2007; Pan et al. 2015; García-Plazaola et al. 2017). In addition, pigment variations can be measured using different non-invasive approaches: (1) delayed fluorescence (Gould et al. 2009), (2) chlorophyll meter (Hoel and Solhaug 1998; Samsone et al. 2007; Mamrutha et al. 2017), and (3) multispectral imaging (Pan et al. 2015). Despite that, only a few species-specific investigations have been done on chlorophyll circadian rhythmicity. For example, Samsone et al. (2007) used a SPAD-502 chlorophyll meter to show that leaf chlorophyll content in bean (*Phaseolus vulgaris*) leaves was lowest in the morning and highest at sunset. Conversely, using the same meter during a natural night-day-night cycle experiment on winter wheat (*Triticum aestivum*), Hoel and Solhaug (1998) found that chlorophyll contents were highest at dawn and dusk and lowest at midday.

Leaf shading treatments can also strongly influence the leaf chlorophyll contents (Lichtenthaler et al. 1981; Dai et al. 2009) and may affect circadian rhythms. Plants grown in the shade can optimize light absorption by increasing chlorophyll density per unit leaf mass (Boardman 1977; Lichtenthaler et al. 1981; Wittmann et al. 2001; Dai et al. 2009). However, previous studies have focused on chlorophyll rhythmicity only in light-exposed leaves (Hoel and Solhaug 1998; Samsone et al. 2007), and to the best of our knowledge, no studies have investigated circadian rhythms in pigment levels in shade-adapted versus sun-exposed plants.

Circadian rhythms and light conditions can also affect other non-photosynthetic leaf pigments such as flavonoids (e.g., flavonols and anthocyanins) (Thain et al. 2002). In leaves, flavonoids, and in particular flavonols (e.g., quercetin), can play a key role as antioxidants for photoprotection (Agati et al. 2020). Anthocyanins differ from other flavonoids for their particular reddish/purplish colouration (Gould et al. 2008; Landi et al. 2015). Anthocyanins absorb mainly in the visible green spectrum (520–560 nm) but also a considerable amount in blue range (450–490 nm) (Landi et al. 2021). This particular feature can induce physiological adjustments in cyanic leaves similar to those in shaded leaves, causing the so-called "shade avoidance syndrome" (Manetas et al. 2003; Landi et al. 2021). Therefore, the presence of anthocyanins may indirectly influence the chlorophyll circadian rhythms due to their photoprotective functions.

Trees, more than herbaceous species, can provide a record of plant rhythmicity through the production of annual woody rings. Usually, young trees grow in the understory, which receives only 0.5%–5% of the sunlight (Chazdon and Pearcy 1991), and their leaves acclimate to the shade (Cao 2000). Moreover, some tree species have red leaves, either constitutively (e.g., in ornamental species) or transiently (i.e., in young or senescing leaves), due to the presence of anthocyanins (Manetas et al. 2003; Hughes et al. 2014; Lo Piccolo et al. 2020, 2021). Therefore, circadian leaf rhythms could be potentially set to maximize light harvesting in different environmental and physiological conditions. Elucidating the pigment dynamics as daily light and light availability (sun versus shade) vary has increase our understanding of the rhythmicity of leaf physiology and more generally of tree performance.

Therefore, here we used an optical leaf-clip sensor to monitor the circadian rhythmicity in the leaf chlorophyll, flavonol and anthocyanin contents in three green-leafed tree species (*Platanus*×*acerifolia*, *Tilia platyphyllos* and *Ailanthus altissima*) and two red-leafed (*Prunus cerasifera* var. *pissardii* and *Acer platanoides* cv. Crimson King) in full sun and in shade. We also explored whether red leaves, rich in anthocyanins in the adaxial epidermis (shading the subjacent mesophyll cells) can screen part of the incident light that reaches the leaf lamina, as shaded leaves do.

Materials and methods

Plant material and experimental conditions

Experiments were conducted in June 2021 at the Department of Agriculture, Food and Environment (DAFE), University of Pisa (43.704672° N, 10.427292° E). Ten 4-year-old trees each of *A. altissima* (Mill.) Swingle (AI), *P.* × *acerifolia* (Aiton) Willd. (PL) and *T. platyphyllos* Scop. (TI) with green leaves, and *P. cerasifera* Ehrh. var. *pissardii* (PR) and *A. platanoides* L. cv. Crimson King (AC) with red leaves were selected and divided into two groups: one group was kept in direct sun (L); the other group was kept in the shade (~ 10 µmol m^{-2} s^{-1}; S) to adapt for 1 week before analyses. Trees were kept well-watered throughout the trials. For each tree, a mature leaf was selected, marked and its pigment levels were monitored throughout a 24-h period. The

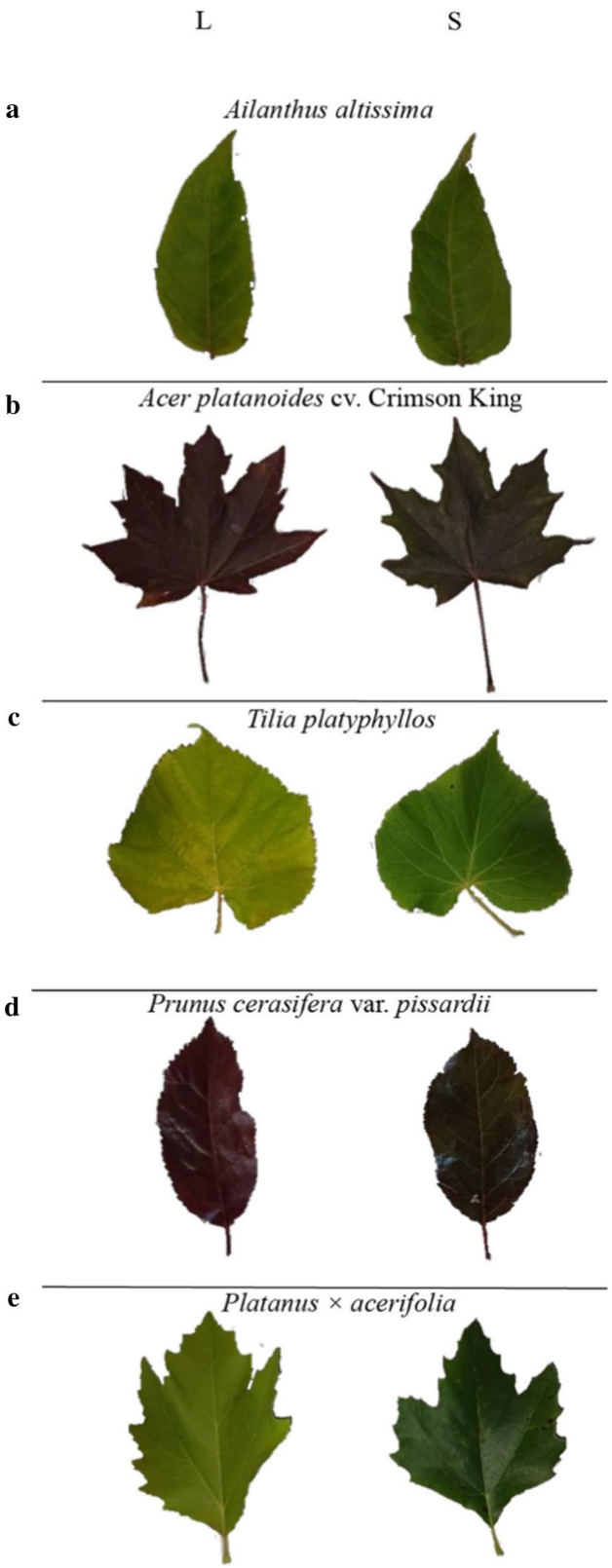


Fig. 1 Leaves from trees in sun and shade for each species. Leaves ▸ of *Ailanthus altissima* **a**, *Acer platanoides* cv. Crimson King **b**, *Tilia platyphyllos* **c**, *Prunus cerasifera* var. *pissardii* **d**, *Platanus*×*acerifolia* and **e** in sun- (L) and shade (S) in June 2021.Fig. 2 Mean (±SD) chlorophyll content in leaves ($n=5$) of trees of five species in the sun and shade measured in June 2021. Sun (L): empty circles; shade (S): full circles. (**a**, **b**) *Ailanthus altissima* (AI), (**c**, **d**) *Acer platanoides* cv. Crimson King (AC), (**e**, **f**) *Tilia platyphyllos* Scop. (TI), (**g**, **h**) *Prunus cerasifera* var. *pissardii* (PR), (**i**, **j**) *Platanus*×*acerifolia* (PL). Means without letters did not differ significantly; means with different letters differed significantly ($P<0.05$) in a one-way ANOVA with different times of day as variability factor, followed by a post hoc Fisher's least significant difference (LSD) test. Some error bars were too small to be seen

experiment was done twice, with similar results, and a representative data set is reported herein.

Pigment detection

Leaf chlorophyll content was measured, and flavonol and anthocyanin indexes were developed using the leaf-clip sensor DUALEX® (Force-A, Orsay, FR). On 30 June, the content in each leaf ($n=5$) was measured between dawn (5:41 GMT) and dusk (21:02 GMT) (i.e. 6:30, 9:00, 10:30, 12:00, 13:30, 15:00, 16:30, 18:00, 19:30 and 21:00). The 50 values (5 measurements/time × 10 times) obtained for each pigment were averaged.

Spectral light quality

At the same time that pigments were measured, spectral quality, light intensity and colour were measured with a portable spectrophotometer (SpectraPen mini, PSI, Drásov, CZ) calibrated for visible light (340 to 850 nm).

Statistical analysis

The 10 means for a pigment were analysed for significant differences over time using a one-way analysis of variance (ANOVA) using the daily sampling time as a source of variation. When a significant difference was found, the means were separated by a post hoc Fisher's least significant difference (LSD) test ($p=0.05$). Homoscedasticity of data was tested using Bartlett's test. The normality of data was tested using D'Agostino & Pearson test. Differences between values for sun and shade were analyzed using an unpaired *t*-test. Linear regressions between photosynthetically active radiation (PAR) values and DUALEX® chlorophyll contents (Chl) were performed; then, R^2 and the *p*-value were established. All statistical analyses were conducted using GraphPad (GraphPad, La Jolla, CA, USA).

Results

Chlorophyll variations

Differences in leaf color between sun- (L) and shade-adapted (S) plants were evident (Fig. 1). Chl content was always significantly higher in the S-plants regardless of species or the species leaf color (+ 23.5, + 24.4, + 84.3, + 42.5 and + 101.5% in AI, AC, TI, PR and PL, respectively; Table 1).

Circadian variations in chlorophyll from 6:30 to 21:00 in L- and S-plants are reported in Fig. 2. Although Chl levels in L-plants generally yielded a flatter curve than that for Chl in shade-adapted leaves, no general trend was observed among L-plants. Red-leafed AC-L and PR-L and the green-leafed PL-L had a significant increase in Chl content at dusk, but levels in green-leafed AI-L and TI-L did not differ significantly across the 10 sampling times. Moreover, in PR-L leaves, Chl values at 9:00 and at 15:00 were significantly lower than at dawn, similar to the trend in S-plants. In the shade, Chl levels had a similar pattern among plants species (Fig. 2). In comparison to values obtained at dawn, Chl values in S-leaves were lowest in the early morning (9:00) and at midday (2:00 and 15:00), and highest at dusk (21:00) (Fig. 2b, d, f, h). This trend was found in each S-plant species (except for PL-S, Fig. 2j).

Flavonol variations

The differences in the flavonol index (Flv) values in the S-plants compared to the L-plants were species-dependent (Table 1). In AI-S and TI-S plants, the Flv index increased by ~ 10%, whereas in AC-S, TI-S and PL-S plants, the Flv index decreased with relative to that for the L-plants (–10.1, 4.3 and 18.6%, respectively).

Circadian Flv trends differed in plant species in relation to L and S treatments (Fig. 3). In AC-L leaves, Flv values varied significantly during the day, showing a decrease at 15:00 and then rose until dusk (Fig. 3c). A similar trend was observed also for PL-L leaves, with the lowest values at 9:00, 12:00 and 15:00 with respect to those at dawn and dusk (Fig. 3i). The Flv values in AI-L, TI-L and PR-L leaves did not vary during the day (Fig. 3a, e, g). In each S-species analyzed, the Flv index did not vary during the day (Figs. 3b, d, f, h, j).

Fig. 2 Mean (± SD) chlorophyll content in leaves (n = 5) of trees of five species in the sun and shade measured in June 2021. Sun (L): empty circles; shade (S): full circles. (**a**, **b**) *Ailanthus altissima* (AI), (**c**, **d**) *Acer platanoides* cv. Crimson King (AC), (**e**, **f**) *Tilia platyphyllos* Scop. (TI), (**g**, **h**) *Prunus cerasifera* var. *pissardii* (PR), (i, j) *Platanus* × *acerifolia* (PL). Means without letters did not differ significantly; means with different letters differed significantly ($p < 0.05$) in a one-way ANOVA with different times of day as variability factor, followed by a post hoc Fisher's least significant difference (LSD) test. Some error bars were too small to be seen.

Anthocyanin variations

Anthocyanin index (Anth) was undetectable in acyanic leaves, and significant differences emerged between the two red-leafed species in the L and S treatments (Table 1). Anth values were much lower in S- than in L-plants (–43.7 in AC, -62.1% in PR). In both red-leafed species, regardless of treatment, the Anth values did not show any significant rhythmicity throughout the day (Fig. 4).

Diurnal light variations

During the day, the light intensity varied among L and S treatments (Table 2). The PAR values were highest from 12:00 to 15:00 in L conditions (~ 1630 µmol m^{-2} s^{-1}) and from 10:30 to 18:00 (~ 10 µmol m^{-2} s^{-1}) in S conditions (Table 2).

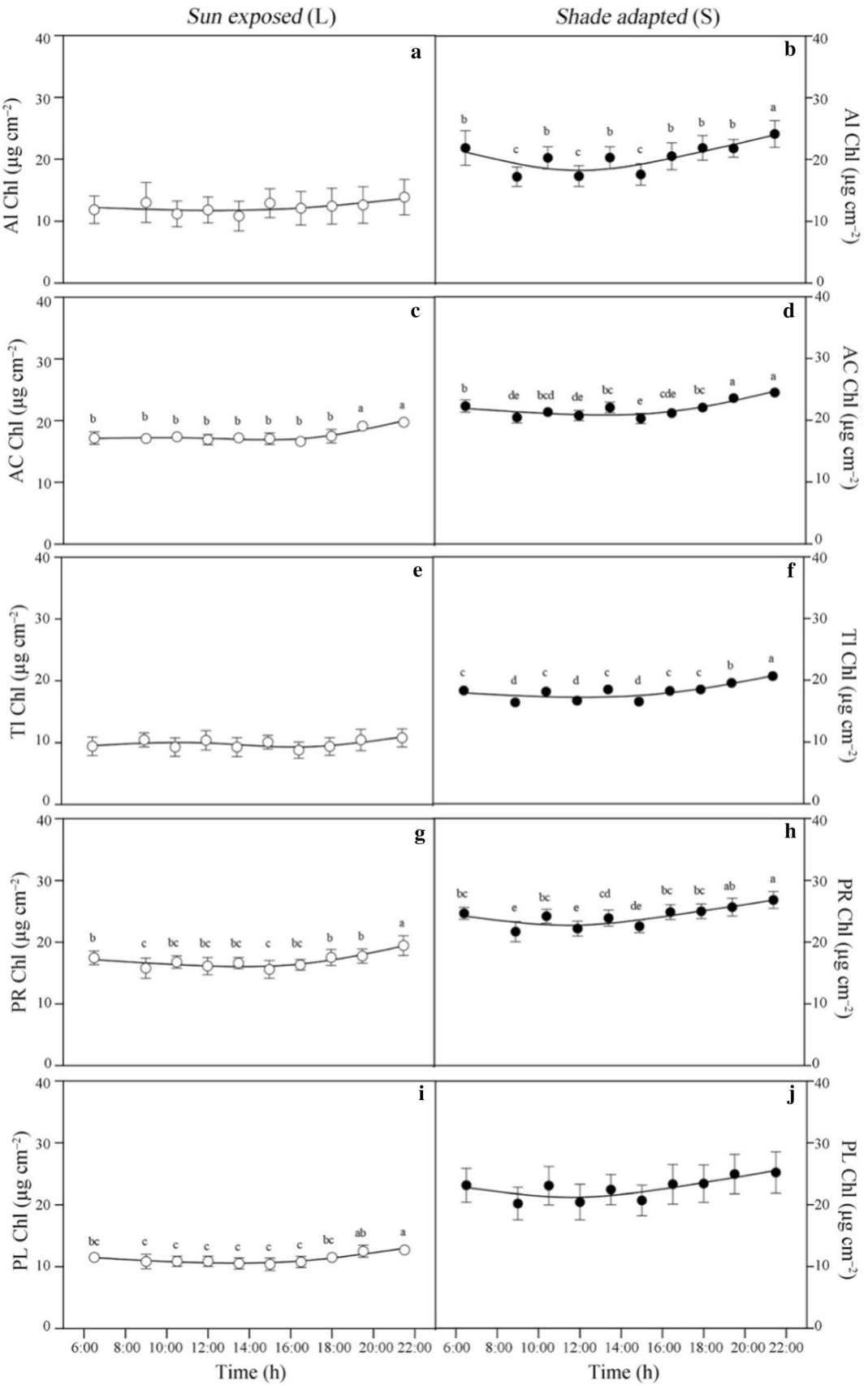
Relationship between PAR and Chl content

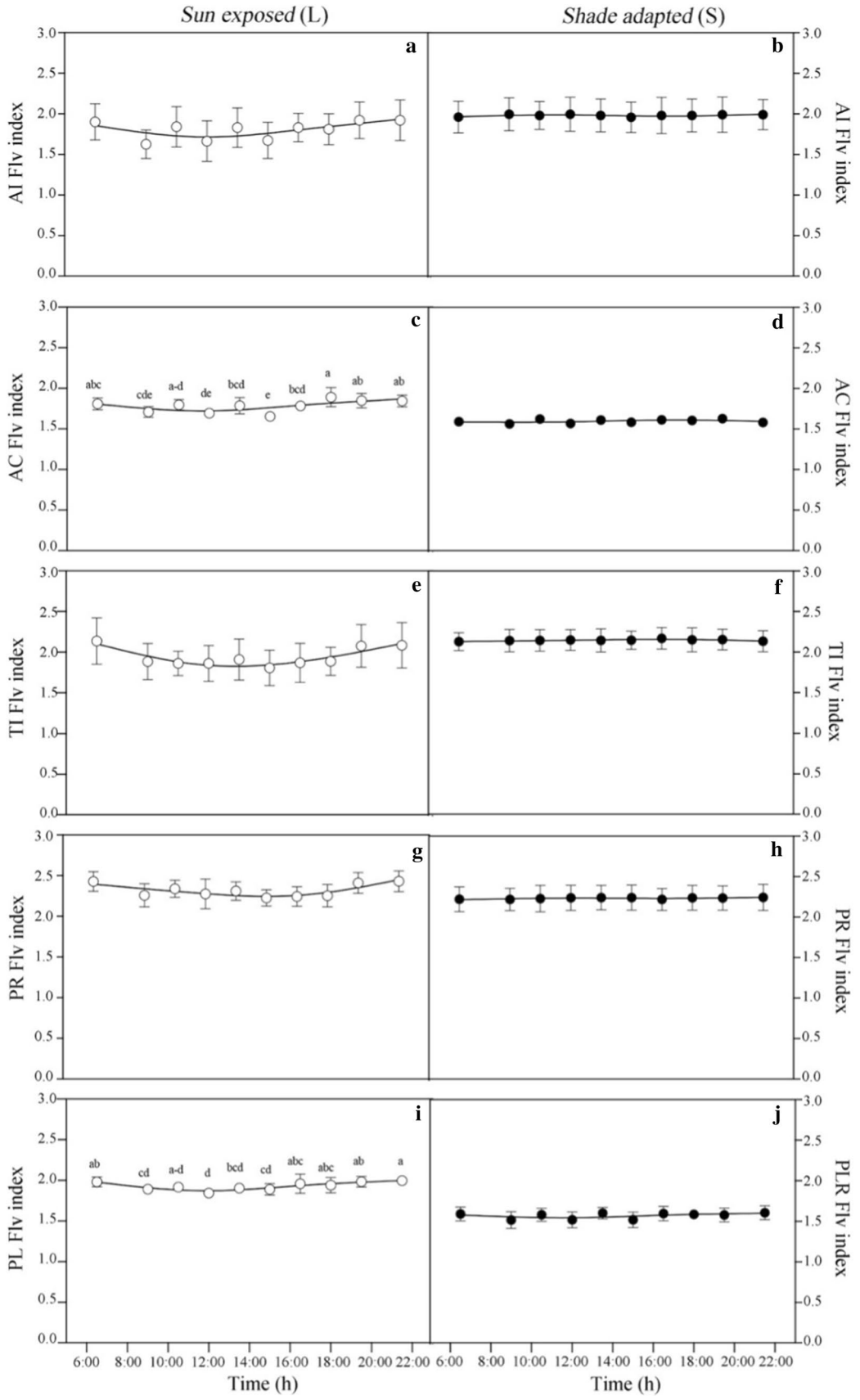
The linear regression analyses between Chl values and relative daily PAR for the L- and S-plants indicated that there was no linear relation for almost all the plants analyzed

Table 1 Means (± SD, n = 50) for chlorophyll content and flavonols and anthocyanin indices in green- and red-leafed tree species in the sun and shade

Species	Chlorophyll (µg cm^{-2})			Flavanol index			Anthocyanin index		
	L	S	*P*	L	S	*P*	L	S	*P*
AI	12.29 ± 2.51	15.18 ± 2.00	***	1.80 ± 0.23	1.98 ± 0.18	***	–	–	
AC	17.59 ± 1.19	21.87 ± 1.49	***	1.78 ± 0.10	1.60 ± 0.04	***	0.48 ± 0.07	0.27 ± 0.02	***
TI	9.86 ± 1.47	18.18 ± 1.42	***	1.94 ± 0.24	2.15 ± 0.12	***	–	–	
PR	16.97 ± 1.59	24.18 ± 1.92	***	2.32 ± 0.14	2.22 ± 0.13	***	0.87 ± 0.17	0.33 ± 0.07	***
PL	11.26 ± 1.10	22.70 ± 3.13	***	1.93 ± 0.08	1.57 ± 0.09	***	–	–	

Notes: AI = *Ailanthus altissima*; AC = *Acer platanoides* cv. Crimson King; TI = *Tilia platyphyllos*; PR = *Prunus cerasifera* var. *pissardii*; PL = *Platanus* × *acerifolia*; L = sun-adapted conditions; S = shade-adapted conditions. Data were analyzed using an unpaired *t*-test (***$P < 0.001$)


Sun exposed (L)
Shade adapted (S)
a
b
c
d
e
f
g
h
i
j
Al Chl (µg cm⁻²)
AC Chl (µg cm⁻²)
Tl Chl (µg cm⁻²)
PR Chl (µg cm⁻²)
PL Chl (µg cm⁻²)
0
10
20
30
40
6:00 8:00 10:00 12:00 14:00 16:00 18:00 20:00 22:00
Time (h)


Sun exposed (L)
Shade adapted (S)
a
b
c
d
e
f
g
h
i
j
AI Flv index
AC Flv index
TI Flv index
PR Flv index
PL Flv index
PLR Flv index
abc cde a-d de bcd e bcd a ab ab
ab cd a-d d bcd cd abc abc ab a
6:00 8:00 10:00 12:00 14:00 16:00 18:00 20:00 22:00
Time (h)

◂**Fig. 3** Hourly trends in flavonol index in in June 2021 in five species of sun- (L; empty circles) and shade-adapted (S; full circles) trees. (**a**, **b**) *Ailanthus altissima* (AI), (**c**, **d**) *Acer platanoides* cv. Crimson King (AC), (**e**, **f**) *Tilia platyphyllos* (TI), (**g**, **h**) *Prunus cerasifera* var. *pissardii* (PR), (**i**, **j**) *Platanus*×*acerifolia* (PL). Each value is the mean ± SD of 5 replicates. Means without letters are not significantly different, means with different letters differed significantly ($p = 0.05$) in one-way ANOVA using the different time of the day as variability factor, followed by mean separation using Fisher's least significant difference (LSD) test. Error bars that appear to be absent were too small to be seen

(Figs. 5a–c, f, h–j). Only for PR-L, PL-L and AC-S was a significant relation found (R^2: 0.59, 0.69 and 0.51, respectively; $p = 0.009$, 0.003, 0.02, respectively; Figs. 5d, e, g).

Discussion

Light quantity and quality influenced the leaf pigment content

Light is well known to have a pivotal role in plant development and rhythmicity. Light signals are perceived by photoreceptors and transduced to the regulatory network, affecting multiple plant physiological traits (Rugnone et al. 2013). Leaf chlorophyll content is a well-accepted reference system to investigate the physiological response of plants to different environmental constraints (Sharma et al. 2020).

Low light conditions induce leaves to optimize their light absorption effectiveness by increasing chlorophyll content per surface unit (Lichtenthaler et al. 1981; Wittmann et al. 2001; Tang et al. 2015). In these experiments, the increase in Chl contents detected in all the shaded species demonstrated the dynamic ability of plants to maximize the light-harvesting capacity in low light. On the other hand, the lower Chl contents detected in the sun-adapted tree leaves is likely aimed to prevent possible photoinhibition of photosynthesis under excessive light, especially around midday (Jason et al. 2004; Dai et al. 2009; Hu et al. 2021).

The synthesis of flavonoids, and in particular, flavonols (e.g., quercetin), in response to increased light radiation (mainly UV-B and blue light) can increase strongly (Agati et al. 2020). Flavonoids, in particular flavonols, are located in different cellular organelles and structures (e.g., vacuoles, cytosol, nucleus, cell wall), and recent pieces of evidence suggest the primary role of these compounds as ROS scavengers instead of a mere light filter (Agati et al. 2007, 2020). In most of the sun-adapted trees analyzed in these experiments (AC, PR and PL), flavonol content was higher than in the shaded counterparts of the same species. However, there was no consistency or dependency between leaf color and flavonol production in the sun, given that two species have red leaves (AC and PR) and one had green (PL). Conceivably, the lack of increase in flavonol content in the sun in the green-leafed AI and TI might be attributable to other biochemical or morphoanatomical features such as the biosynthesis of alternative UV-absorbing/ROS scavenging compounds (Karabourniotis et al. 2020).

Anthocyanins are localized in cell vacuoles and synthesized in different plant organs and tissues (Gould et al. 2008). In the analyzed species (*A. platanoides* cv. Crimson King and *P. cerasifera* var. *pissardii*), anthocyanins are localized in leaf epidermal (adaxial and abaxial epidermis) and mesophyll cells (data not shown). Light (quality and quantity) is one of the key regulators for inducing anthocyanin synthesis in plant organs and tissues (Das et al. 2012). For example, in purple pak-choi plants subjected to low light conditions, enzymes involved in the anthocyanin biosynthetic pathway (chalcone synthase, chalcone isomerase and

Table 2 Photosynthetically active radiation (PAR) and intensity of blue, green, yellow, red and far red light from dawn to dusk in full sun (L) and in shade (S)

Time	PAR		Blue light		Green light		Yellow light		Red light		Far Red light	
	(μmol m^{-2} s^{-1})		(μmol m^{-2} s^{-1})		(μmol m^{-2} s^{-1})		(μmol m^{-2} s^{-1})		(μmol m^{-2} s^{-1})		(μmol m^{-2} s^{-1})	
	L	S	L	S	L	S	L	S	L	S	L	S
06:30	36.2	0.3	12.2	0.1	8.6	0.1	3.8	0	11.6	0.1	10.6	0.2
09:00	739.1	5.8	190.2	1	172.2	1.4	95.8	0.8	281	2.6	260.8	5
10:30	848.8	9.9	226.1	1.9	198.1	2.5	110.1	1.4	314.5	4.1	298.7	8.3
12:00	1705.9	10	440.1	1.8	398.8	2.4	225.7	1.5	641.3	4.4	528.7	7.5
13:30	1667.1	8.8	442.4	1.7	388.8	2.1	217.4	1.3	618.6	3.8	552.1	6.9
15:00	1637.7	10.1	429.2	1.9	381.7	2.4	214.2	1.4	612.6	4.4	552.4	8.1
16:30	1104	10.9	287.8	2	257.3	2.6	143.6	1.5	415.4	4.7	374.9	8.2
18:00	195.3	11.2	74	1.9	47.9	2.7	21.8	1.6	51.5	5.1	43.4	7.8
19:30	99.4	1.6	36.9	0.4	24.3	0.4	10.9	0.2	27.3	0.6	24.2	1.1
21:00	–	–	–	–	–	–	–	–	–	–	–	–

flavanone 3 hydroxylase) are inhibited, causing the purple leaves to turn green (Zhu et al. 2017). Our results are consistent with this decrease in foliar anthocyanins in pak-choi in low light (Zhu et al. 2017). However, since there is a strong link between anthocyanins and leaf sugar content (Das et al. 2012; Lo Piccolo et al. 2020; Ichimura et al. 2021), we cannot exclude that, the very low light conditions in our experiments (Table 2) affected leaf photosynthesis, reducing the available carbon skeletons to synthesize secondary metabolites such as flavonoids involved in plant–environment interactions.

Pigment circadian rhythms in sun and shade

Nondestructive analyses that use spectral wavebands are an effective tool for plant monitoring (including circadian rhythmicity) and provide a broad range of physiological information on plant status (Hoel and Solhaug 1998; Samsone et al. 2007; Pan et al. 2015; Mamrutha et al. 2017). However, Hoel and Solhaug (1998) raised doubts about the efficacy of chlorophyll meters to measure diurnal chlorophyll variations since natural irradiance may affect chlorophyll meter readings. Indeed, Kasahara et al. (2002) increased the light irradiance and observed light-dependent chloroplast movements, which were likely induced to reduce photodamage. These movements, in turn, change the leaf absorbance, and therefore could also affect Chl values obtained from the chlorophyll

Fig. 5 Linear regressions between photosynthetically active radiation (PAR) and DUALEX® chlorophyll content (Chl) in June in sun- (L) and shade-adapted (S) trees five species. (**a**, **f**) *Ailanthus altissima* (AI), (**b**, **g**) *Acer platanoides* cv. Crimson King (AC), (**c**, **h**) *Tilia platyphyllos* (TI), (**d**, **i**) *Prunus cerasifera* var. *pissardii* (PR), (**e**, **j**) *Platanus*×*acerifolia* (PL). * $P<0.05$, ** $P<0.01$

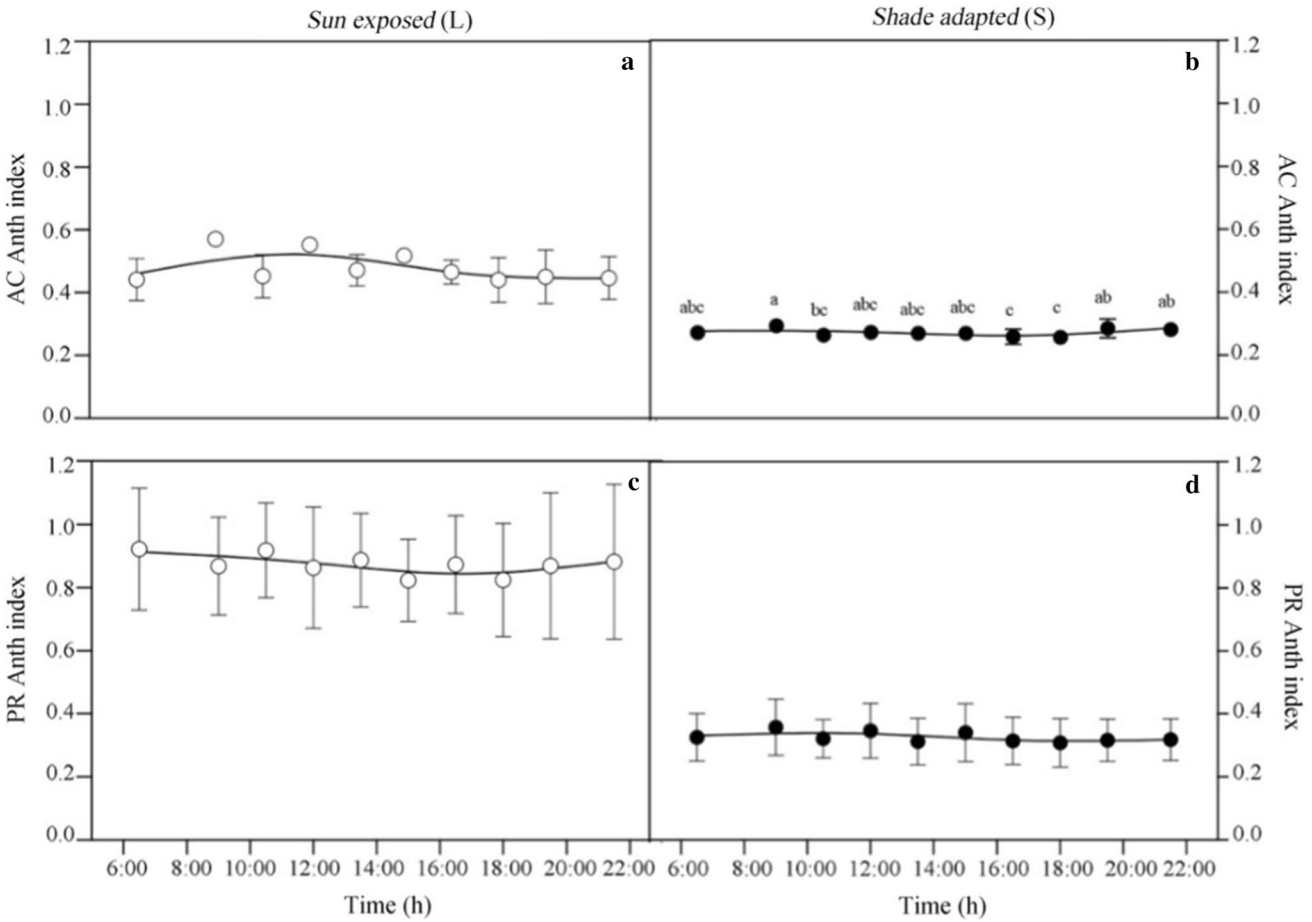
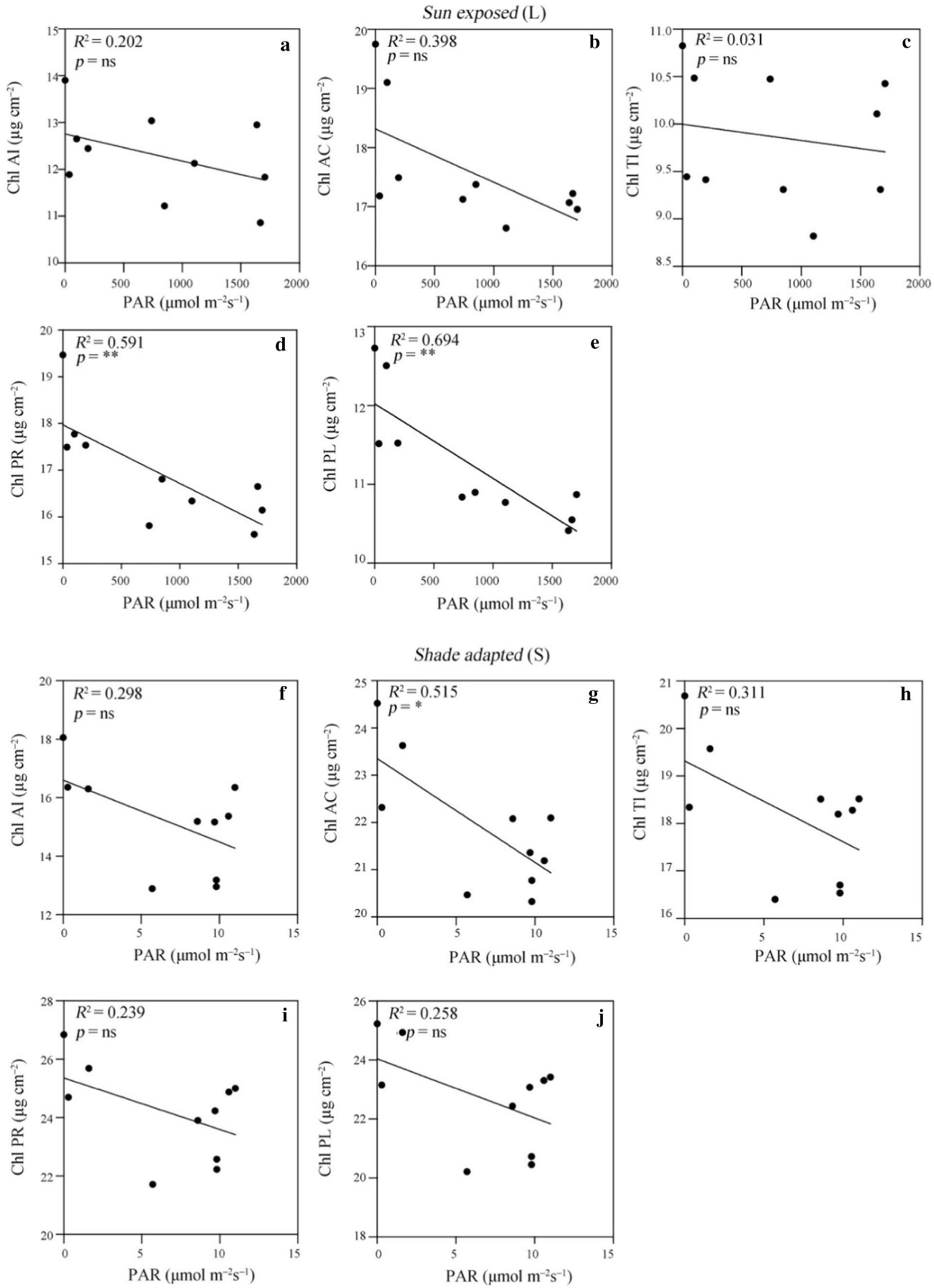


Fig. 4 Hourly trends in the anthocyanin index in June 2021 in sun- (L; empty circles) and shade-adapted (S; full circles) trees of two red-leafed species. (**a**, **b**) *Acer platanoides* cv. Crimson King (AC) and (**c**, **d**) *Prunus cerasifera* var. *pissardii* (PR). Each value is the mean ± SD of 5 replicates. Means without letters are not significantly different, means with different letters differed significantly ($p=0.05$) in one-way ANOVA using the different time of the day as variability factor, followed by mean separation using Fisher's least significant difference (LSD) test. Error bars that appear to be absent were too small to be seen


Sun exposed (L)
a
$R^2 = 0.202$
p = ns
Chl AI (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
b
$R^2 = 0.398$
p = ns
Chl AC (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
c
$R^2 = 0.031$
p = ns
Chl TI (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
d
$R^2 = 0.591$
p = **
Chl PR (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
e
$R^2 = 0.694$
p = **
Chl PL (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
Shade adapted (S)
f
$R^2 = 0.298$
p = ns
Chl AI (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
g
$R^2 = 0.515$
p = *
Chl AC (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
h
$R^2 = 0.311$
p = ns
Chl TI (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
i
$R^2 = 0.239$
p = ns
Chl PR (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})
j
$R^2 = 0.258$
p = ns
Chl PL (µg cm^{-2})
PAR (µmol m^{-2}s^{-1})

meter (Hoel and Solhaug 1998). However, our data indicated either no significance or very low significance (only in PR and PL) in the relation between PAR and Chl content measured with the leaf-clip sensor in sun-adapted leaves (Fig. 5).

Moreover, in the present experiment, the results from S-plants grown at a maximum PAR of ~ 10 µmol m^{-2} s^{-1} and those from those shaded individuals disentangled this potential problem. Therefore, we can reasonably assume that the instrument used in the analyzed species was not influenced by diurnal variation in light irradiance in the current study. Our findings are in accordance with Mamrutha et al. (2017), reported that the chlorophyll meter used in the present study provided reliable chlorophyll values for monitoring leaf chlorophyll. The use of the nondestructive DUALEX® meter allowed us, for the first time, to "live" monitoring of simultaneous changes in chlorophyll, flavonol and anthocyanin circadian rhythms.

Molecular studies in *A. thaliana* have shown that multiple levels of regulation modulate the biosynthesis of chlorophylls. Transcriptome profiles showed that chlorophyll biosynthetic genes could be grouped into different gene clusters based on their response to light and circadian clock rhythms (Matsumoto et al. 2004; Kobayashi and Masuda 2019). Molecular regulation of the genes leads to the rhythms of chlorophyll biosynthesis, downregulating synthesis at night and upregulating synthesis during the day (Kobayashi and Masuda 2019). Our results agree with previous molecular analyses that suggest chlorophyll is not synthesized at night because no increase in chlorophyll was detected at dawn in any analyzed species. Moreover, chlorophylls undergo a continuous daily breakdown/biosynthesis turnover, which can be adjusted to accommodate external light conditions and fluctuations (Matile et al. 1999; Hu et al. 2021). When chlorophyll turnover is at a steady-state, no changes in chlorophyll content are detected due an equilibrium between chlorophyll anabolism and catabolism (Matile et al. 1999). Therefore, we hypothesize that the trends in leaf chlorophyll content, detected especially in shade-adapted leaves, can be explained by a different rate in chlorophyll biosynthesis and degradation during the whole day. These trends were less visible in sun-exposed leaves, probably because the higher light conditions compared to the shading of the S-leaves, reduced the chlorophyll content, masking the increased values registered for the shaded counterparts during the late afternoon.

The circadian regulation of primary metabolism has been extensively studied, but work on the circadian regulation of secondary metabolism, such as flavonoid metabolism, is still limited (Thain et al. 2002; Soengas et al. 2018). Working with different plant species, Gori et al. (2020) and Veit et al. (1996) reported that the total flavonoid content increased during the central hours of the day, probably to reduce the potential oxidative stress due to excessive light. However, our experiments unveiled very few changes in daily flavonol levels, with the flavonol indices at dawn similar to those at dusk (Fig. 3). The almost unchanging daily trend in the flavonol index in all the analyzed tree species suggests that light conditions during the day were not so excessive that antioxidants were transiently induced in the plants.

Although anthocyanins showed no significant daily trends in levels in red-leafed plants in both L and S, their presence indirectly influenced the content and daily variation in chlorophylls. In our study, in both red-leafed species species, sun-exposed leaves had higher Chl values than those in all the green-leafed species (Table 1; $P < 0.0001$, Student's *t*-test). The daily trend in chlorophyll levels in sun-exposed leaves of PR was very similar to that obtained for the shaded counterpart; they were significantly lower at 9:00 and 15:00 than those at dawn (Fig. 2). Not by chance, among the red-leafed species, PR-L had highest values for the Anth index (Table 1; $P < 0.0001$, Student's *t*-test). Indeed, anthocyanins absorb mostly in the visible green spectrum (520–560 nm) with an appreciable absorbance in blue wavebands (450–490) (Landi et al. 2021). Furthermore red-leafed species usually have several shade-like traits (e.g., reduced leaf thickness, higher chlorophyll content and lower chlorophyll *a*/*b* ratio), suggesting that the presence of epidermal anthocyanins can induce the so-called "shade syndrome" (Manetas et al. 2003; Hughes et al. 2014; Tattini et al. 2014; Landi et al. 2021). Therefore, in our experiment, the sunscreen effect exerted by anthocyanins, partially "shadowing" mesophyll cells, has likely induced the behavior of shaded leaves in sun-exposed red leaves to, at least in terms of Chl daily rhythmicity.

Conclusions

Our results support the use of non-destructive analyses to measure trends in chlorophyll, flavonol and anthocyanin pigment content. The DUALEX® provides a rapid, non-invasive, robust and reliable assessment of pigment indexes, which can be applied in various branches of plant science. Chlorophyll contents and daily variations were influenced by light conditions and anthocyanin presence. Conversely, flavonol daily rhythms did not change significantly. This study provides the basis for further investigations related to pigment rhythmicity in plants, a hot topic in plant physiology, ecology and applied plant sciences such as plant breeding to improve plant fitness in sunny and shady environments.

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