

Seasonal variation in the reflectance of photosynthetically active radiation from epicuticular waxes of Scots pine (*Pinus sylvestris*) needles

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Epicuticular waxes influence leaf reflectance, but the spatiotemporal dynamics in their reflectance properties have not been properly characterized, and its consequences remain unknown. In this study, we analysed the seasonal changes in wax reflectance of Scots pine needles. It tended to decrease with needle age and towards lower positions within the canopy. In addition, we also identified a clear seasonal pattern of variation superimposed on the of above-mentioned wax weathering effect. We conclude that spatiotemporal dynamics in wax optical properties need to be considered in studies that implicitly assume constant light absorption, particularly when different leaf age classes, canopy positions or seasons are compared, and especially in species with substantial amount of waxes. We suggest that the observed dynamics in wax reflectance could represent a new photoprotective mechanism operating at the seasonal scale as they modulate the absorption of the photosynthetically active radiation (PAR) over time.

Introduction

Photosynthesis is the process by which atmospheric carbon dioxide (CO₂) is transformed into sugars using sunlight as energy source and water as electron donor. Photosynthesis involves two different sets of reactions: light and carbon reactions. Light reactions are involved in the absorption of light and production of ATP and NADPH, whereas in the carbon reactions the atmospheric CO₂ is assimilated into sugars using the energy provided by ATP and NADPH. Importantly, photosynthetic energy production and consumption

need to be in balance in order to avoid photodamage caused by excess light (Huner *et al.* 2003). Keeping the photosynthetic machinery energetically balanced is a challenge for plants because, whilst the energy input into the light reactions is a function of overall PAR absorption (mainly modulated by concentration of photosynthetic pigments) and ultimately PAR intensity, the energy consumption by the carbon reactions is strongly dependent on temperature, water status, and the temporal physiological dynamics of the plant.

In order to maintain the photosynthetic energy balance, leaves have mechanisms to modulate the

absorption of radiation and the capacity to harmlessly dissipate the energy absorbed in excess. In the short term, many plants species change the orientation of leaves (Muraoka *et al.* 1998) and reposition chloroplasts inside the cells (Kasahara *et al.* 2002) to minimize harmful quantities of light. Non-photosynthetic pigments within cells, such as anthocyanins and flavonoids, can also reduce the amount of light available to photosynthesis (Chalker-Scott 1999, Han *et al.* 2003). In addition to adjustments in the quantity of absorbed light, plants can also alleviate the problem by regulating the fraction of absorbed excitation energy that is thermally dissipated, via the xanthophyll-cycle reactions and the protonation/de-protonation of specific pigment-binding proteins (Gilmore 1997, Müller *et al.* 2001). In the long term, leaf-level seasonal changes in chlorophyll contents and sustained forms of non-photochemical quenching modulate light absorption and the fraction of thermal energy dissipation, respectively (Öquist and Huner 2003, Porcar-Castell 2011).

In addition, plants can also adjust light absorption by modulating leaf reflectance properties. Salt deposits (Mooney *et al.* 1977, Esteban *et al.* 2013) and trichomes (Holmes and Keiller 2002) found on the leaf surface of many plants have been shown to increase the fraction of reflected light. Similarly, the waxes protruding from the epidermis have been shown to be effective in reflecting radiation (Reicosky and Hanover 1978, Holmes and Keiller 2002, Pfündel *et al.* 2006). Although PAR reflectance of leaves is usually 5%–10% of the incident radiation, in plants with denser wax cover it may be as high as 50% (Mulroy 1979). Thus, variations in the reflectance of epicuticular waxes and its temporal dynamics may have a relevant functional role in the modulation of light absorption.

PAR reflectance of epicuticular waxes has been analysed in some herbaceous plants and tree species. These studies have focused on comparing species of different ecological groups (Billings and Morris 1951), juvenile and mature leaves of certain species (Cameron 1970), and specimens of the same species with and without glaucous coating (Reicosky and Hanover 1978, Mulroy 1979), yet the temporal dynamics remain unclear. In this study, we examined spatiotemporal dynamics of PAR reflectance of epicuticular

waxes during the course of a year in different needle ages within the canopy of boreal Scots pine trees growing under natural conditions. We hypothesized that, in addition to the reduction in reflectance caused by natural wax weathering, there is also a seasonal reflectance pattern, which could contribute to reducing the excitation pressure in leaves and protect them from photo-damage.

Material and methods

Experimental design

Eight 49-year-old Scots pines (*Pinus sylvestris*) growing at SMEAR II (Station for Measuring Ecosystem–Atmosphere Relations; see Hari and Kulmala 2005 for more details) in southern Finland (61°31'N, 24°17'E, 180 m a.s.l.) were selected for repeated reflectance measurements, and accessed using permanent scaffold towers. The measurements were carried out from August 2010 to June 2011 in four campaigns: late summer (19–20 August 2010), autumn (22–24 November 2010), winter (21–23 February 2011) and early summer (9–11 June 2011). The measurements were done at two positions within the canopy: top-canopy (ca. 15 m), where the needle samples were collected from the three uppermost living whorls, and low-canopy (ca. 7 m), where the needle samples were collected from the three lowest living whorls. In each of the canopy positions, the measurements were made for the three available needle ages. For each of the trees, the measurements made per canopy position and needle age were repeated four times.

All the PAR reflectance measurements were done using detached needles and at room temperature in a cabin located in the vicinity of the study site. The spectral reflectance measurements were performed within 1.5 hours after detaching the needles. Each reflectance spectra was recorded from three to four pairs of needles using a portable spectroradiometer (ASD Inc. FieldSpec® HH VIS-NIR, USA), spectral range 325–1075 nm, spectral resolution FWHM (full width half maximum) of 3.5 nm, and sampling interval of 1.6 nm, in combination with a plant probe (viewing angle of 45°) with halogen light source and a fiber optic

(ASD Inc., USA). The needles were arranged in a dark-acclimation leaf clip designed for chlorophyll measurements (Hansatech Ltd., UK), but used in this study to get a reduced and constant needle area for the plant probe (Porcar-Castell *et al.* 2012). The clip fitted perfectly the plant probe. Reflectance spectra were recorded by an integration time of 544 ms, each spectrum being averaged over ten measurements. Measurements were repeated until a steady reflectance reading was obtained. Typically, the third spectral average was stable and recorded, within 20 seconds. Reference measurements with a Spectralon® reflectance standard and dark current correction were performed every eight recordings.

In order to study the effect that epicuticular waxes have on needle reflectance, spectral measurements were conducted both before and after removing the epicuticular waxes by dipping the needles in a 99.8% chloroform bath for 10 seconds. As the chloroform bath time can alter the amount of chlorophylls (Bewick *et al.* 1993), differences in the optical properties of needles could be due not only to epicuticular waxes but also chlorophyll bleaching. To study the effects that the chloroform bath time has on needle reflectance, we analysed PAR reflectance differences in current-year needles of blue spruce (*Picea pungens*) exposed to chloroform bath times ranging from one second to five minutes. Blue spruce was used as the reference conifer to validate the methodologies used in this study as it represents a conifer species with high amount of epicuticular waxes. We found no differences in PAR reflectance of blue spruce needles in response to the chloroform bath times (data not shown), suggesting that our chloroform bath treatment did not cause any chlorophyll bleaching.

The difference in reflectance spectra caused by the removal of the epicuticular wax layer, hereinafter denoted as $\Delta R(\lambda)$, was calculated as the difference between the needle reflectance spectra of the intact leaf, $R_w(\lambda)$, and that after wax extraction, $R_0(\lambda)$, (Fig. 1):

$$\Delta R(\lambda) = R_w(\lambda) - R_0(\lambda) \quad (1)$$

The effect of the wax layer on PAR reflectance, $\Delta R(\text{PAR})$, was calculated as the average $\Delta R(\lambda)$ for the wavelength range 400 to 700 nm.

The changes in the reflectance induced by wax removal were measured in a single direction using a plant probe, called here $\Delta R(\text{PAR})$, yet we wanted to examine the effect of wax removal on total hemispherical reflectance (*see* Schaepman-Strub *et al.* 2006 for more details). In order to test whether $\Delta R(\text{PAR})$ was a good proxy of differences in hemispherical reflectance, we compared changes in reflectance upon wax removal using the plant probe with changes in total hemispherical reflectance upon wax removal using an integrating sphere (ASD RTS-3ZC, ASD Inc., USA) with current-year needles of blue spruce and Scots pine. The comparison of methods was repeated five times for each of the species with each of the devices, following the wax extraction procedure previously explained.

Due to the unavoidable gap fraction between needles inherent to the integrating sphere measurements (Daughtry *et al.* 1989), we designed holders for the needle samples, thus keeping the same area before and after the wax removal and allowing the calculation of the gap fraction. The needle samples were scanned while still in the holder using a desktop scanner (Agfa Arcus 1200) in grayscale and at a resolution of 800 dpi. The gap fraction between needles was calculated by image processing using Adobe Photoshop CS6, and the hemispherical reflectance was calculated as in Mesarch *et al.* (1999). The Kolmogorov-Smirnov test showed that the data were normally distributed. Using a *t*-test, we did not find significant differences in $\Delta R(\text{PAR})$ neither in Scots pine (plant probe 0.036 ± 0.015 ; integrating sphere 0.045 ± 0.014 , mean \pm SD; *t*-test: $t_8 = 0.941$, $p = 0.374$) nor in blue spruce (plant probe 0.101 ± 0.014 ; integrating sphere 0.104 ± 0.022 , mean \pm SD; *t*-test $t_9 = 0.330$, $p = 0.749$). We conclude that the directional reflectance of the epicuticular waxes estimated by a plant probe was a good proxy for the hemispherical reflectance of epicuticular waxes.

Meteorological data

During the study period, the air temperatures in the top canopy and low canopy were measured with 4-wired PT-100 sensors in a measuring mast at 8.4 m and 16.8 m above ground. The

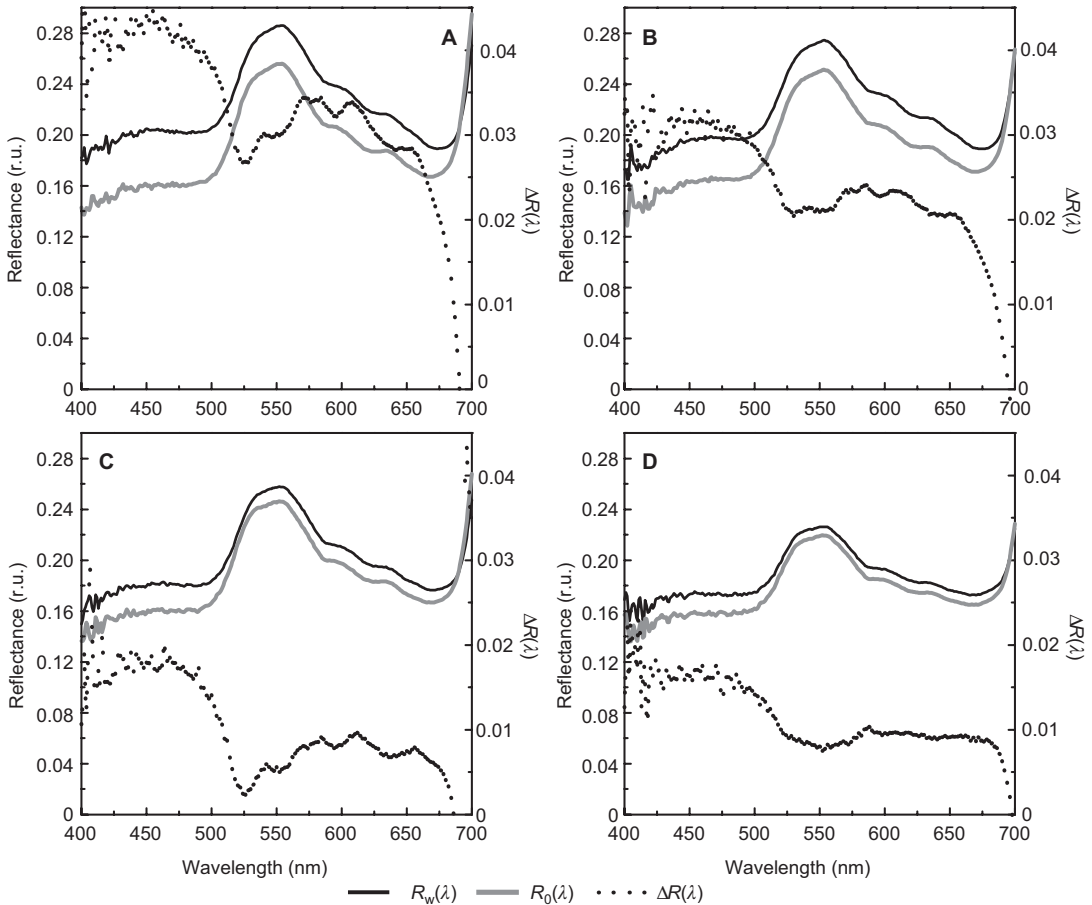


Fig. 1. Late summer PAR reflectance spectra from needles of Scots pine before [$R_w(\lambda)$] and after [$R_0(\lambda)$] epicuticular wax removal, and epicuticular wax PAR reflectance, [$\Delta R(\lambda)$]. (A) Top-canopy and current-year needles (2010), (B) top-canopy and one-year-old needles (2009), (C) low-canopy and current-year needles (2010), and (D) low-canopy and one-year-old needles (2009). Each spectrum is the average of eight replicates.

sensors were protected from solar radiation and ventilated by home-made fans. The total PAR for the top-canopy position was recorded by a LI-190sz quantum sensor (Li-Cor, Lincoln, NE, USA) in the same mast at 18 m above ground, whilst radiation at the low-canopy position was recorded from four additional quantum sensors installed at 0.6 m above the forest ground within the study site.

Statistical analysis

The Kolmogorov-Smirnov test of normality was employed on the $\Delta R(\text{PAR})$ values for each of the factors evaluated: seasonality ($n = 48$), needle age ($n = 64$), and canopy position ($n = 96$), for a

total sample size of 192. The data were normally distributed for each of the factors, hence we proceeded to use parametric tests to assess differences among the factors. A three-way analysis of variance (ANOVA) for seasonality, needle age, and canopy position was conducted, followed by a posterior two-way ANOVA for each of the canopy positions analysed. Along with the two-way ANOVA, *post hoc* Tukey's test was also conducted. The statistical analysis was done using IBM SPSS Statistics (ver. 21).

Results

During the experimental period August 2010–June 2011, no differences were found in the daily

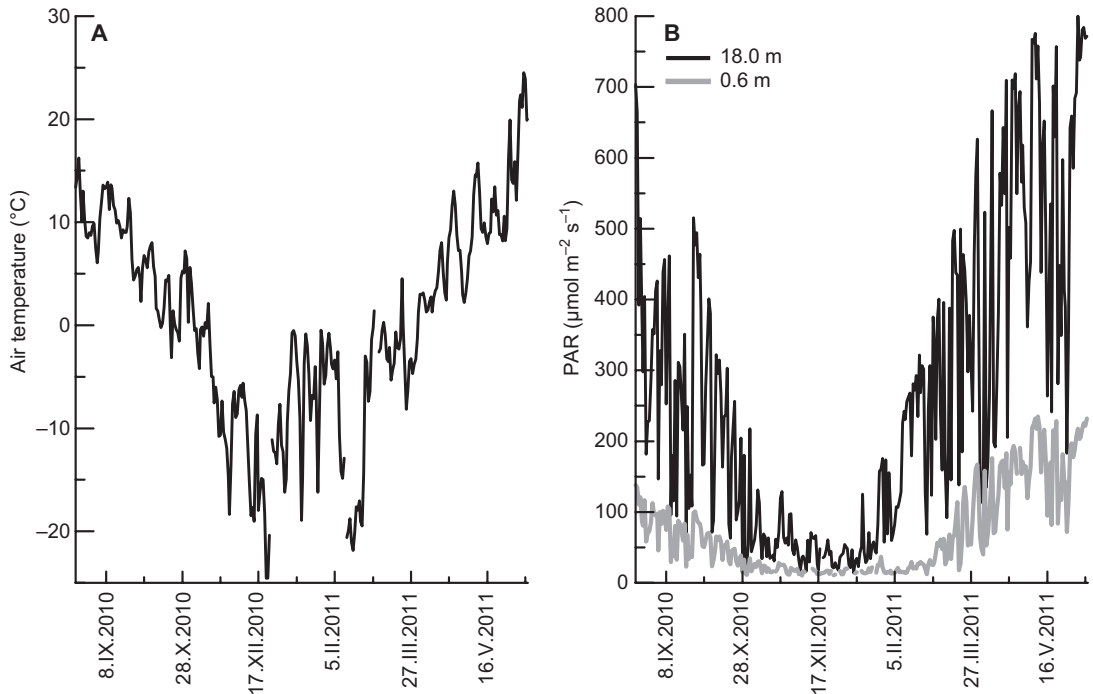


Fig. 2. Daily averaged (A) air temperature ($^{\circ}\text{C}$) within the canopy of a Scots pine forest, and (B) photosynthetically active radiation (PAR, $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 18 m (black line) and 0.6 m (gray line) above the ground of a Scots pine forest, during the study period 19 August 2010 to 11 June 2011.

mean temperature between the top-canopy and the low-canopy, but a clear difference in the light environment was detected (Fig. 2).

The $\Delta R(\text{PAR})$ values for top-canopy were higher than the values for the low-canopy for the same season and needle age (Figs. 1 and 3). Accordingly, the highest $\Delta R(\text{PAR})$ value was registered for top-canopy current-year needles in late summer (0.032 ± 0.004 , mean \pm SE). In the low-canopy, the current-year needles also had the highest $\Delta R(\text{PAR})$ value, but those values were obtained during autumn instead of late summer (0.018 ± 0.004 , mean \pm SE). Regardless of canopy position, the effect of waxes on reflectance was found to decrease with needle age, but the needles with the highest $\Delta R(\text{PAR})$ values (i.e. current- and one-year-old needles from the top canopy) also revealed a seasonal variation.

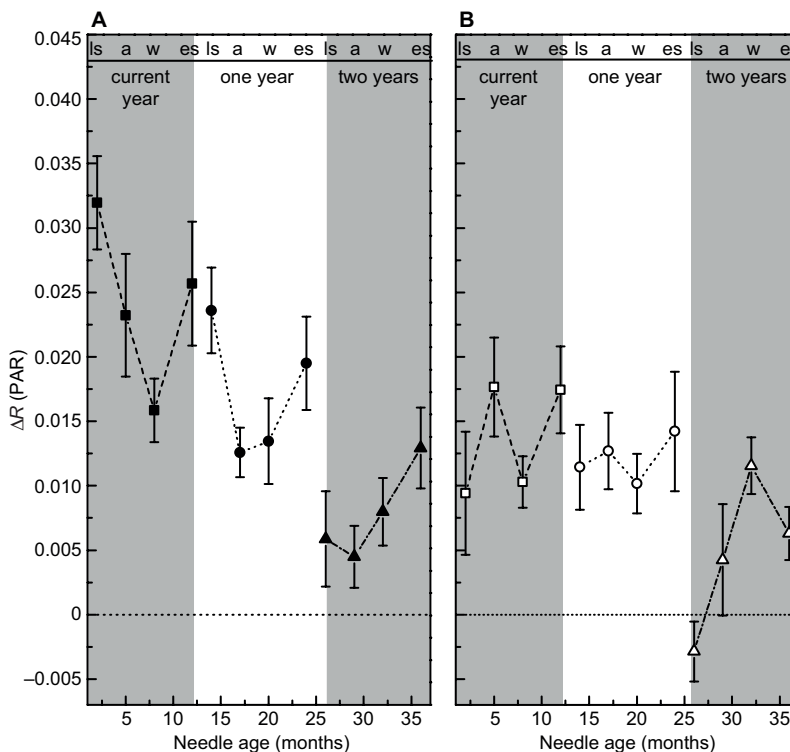
The results of a three-way ANOVA revealed that needle age (three-way ANOVA needle age: $F_{2,192} = 27.818$, $p < 0.001$) and canopy position (three-way ANOVA canopy position: $F_{1,192} = 17.925$, $p = 0.000$) were the most significant fac-

tors affecting $\Delta R(\text{PAR})$ (Table 1). Seasonality was not a significant factor (three-way ANOVA seasonality: $F_{3,192} = 2.243$, $p = 0.085$). However, there was significant interaction between seasonality and both needle age (three-way ANOVA seasonality \times needle age: $F_{6,192} = 2.626$, $p = 0.018$), and canopy position (three-way ANOVA seasonality \times canopy position: $F_{3,192} = 4.715$, $p = 0.003$), but there was no significant interaction between needle age and canopy position (three-way ANOVA seasonality \times canopy position: $F_{2,192} = 2.389$, $p = 0.095$).

In order to disclose whether seasons might have an effect on top- or low-canopy foliage, we analysed the variation in $\Delta R(\text{PAR})$ independently for each canopy position.

A two-way ANOVA with $\Delta R(\text{PAR})$ values from top-canopy position (Table 2) confirmed significant differences in its response to needle age (two-way ANOVA needle age: $F_{2,96} = 22.054$, $p < 0.001$) in any season. The Tukey *post hoc* test revealed that there were significant differences in $\Delta R(\text{PAR})$ between needles of dif-

Fig. 3. $\Delta R(\text{PAR})$ values for (A) top-canopy and (B) low-canopy Scots pine needles. Needles were sampled during the year 2010–2011 but are separated by needle age [current-year needles (2010), one-year-old needles (2009), and two-year-old needles (2008)], and presented in each of the panels as a continuum distinguished by stripes of alternative colours (gray/white/gray). The four points in each of the stripes represent the four sampling seasons [ls = late summer (2010), a = autumn (2010), w = winter (2010), and es = early summer (2010)]. Values are means \pm SEs ($n = 8$).



ferent ages ($p = 0.034$ between current- and one-year-old needles; $p < 0.001$ between current- and two-year-old needles; $p < 0.001$ between one- and two-year-old needles), current-year needles having the highest $\Delta R(\text{PAR})$ values and two-year-old needles the lowest one regardless of the season. Here, significant differences were also found in response to seasonality (two-way ANOVA seasonality: $F_{3,96} = 4.055$, $p = 0.010$),

Table 1. Results from a three-way analysis of variance (ANOVA) comparing age, season, and position in Scots pine needle $\Delta R(\text{PAR})$ values. Data shown below represents degrees of freedom (df), mean squares (MS), and significance levels for each factor and interaction.

Source of variation	df	MS	Significance level
Season	3	0.000	ns
Age	2	0.003	$p < 0.001$
Position	1	0.002	$p < 0.001$
Season \times age	6	0.000	$p < 0.05$
Season \times position	3	0.000	$p < 0.01$
Age \times position	2	0.000	ns
Season \times age \times position	6	4.035E-005	ns
Residual	168	9.088E-005	

with a characteristic increase in $\Delta R(\text{PAR})$ values from winter towards summer in current- and one-year-old needles. The Tukey test revealed that the main difference in $\Delta R(\text{PAR})$ was found between late summer and winter values ($p = 0.025$). The interaction between seasonality and needle age was not significant (two-way ANOVA

Table 2. Two-way analysis of variance (ANOVA) results for Scots pine needle $\Delta R(\text{PAR})$ values for top- and low-canopy, respectively. Data shown below represents degrees of freedom (df), mean squares (MS), and significance levels for each factor and interaction.

Source of variation	df	MS	Significance level
Top-canopy			
Season	3	0.000	$p < 0.01$
Age	2	0.002	$p < 0.001$
Season \times age	6	0.000	ns
Residual	84	9.336E-005	
Low-canopy			
Season	3	0.000	$p < 0.05$
Age	2	0.001	$p < 0.001$
Season \times age	6	0.000	ns
Residual	84	8.839E-005	

seasonality \times needle age: $F_{6,96} = 1.608$, $p = 0.155$).

The effects of age and seasonality on $\Delta R(\text{PAR})$ were similar in the low-canopy and top-canopy positions (two-way ANOVA needle age: $F_{2,96} = 7.763$, $p = 0.001$; two-way ANOVA seasonality: $F_{3,96} = 2.870$, $p = 0.041$). However, current- and one-year-old needles did not differ from each other ($p = 0.813$), and the differences in seasonality were due to differences between late summer and early summer ($p = 0.028$). No interaction between seasonality and needle age was found in $\Delta R(\text{PAR})$ (two-way ANOVA seasonality \times needle age: $F_{6,96} = 1.458$, $p = 0.203$). Thus, although in the three-way ANOVA the interaction between seasonality and needle age was significant, this interaction was not detected when conducting two-way ANOVA, suggesting that the latter interaction was dependent on the canopy position.

Discussion

We investigated seasonal variation in the PAR reflectance of epicuticular waxes of Scots pine, $\Delta R(\text{PAR})$, for different needle ages within the canopy, and found that needle age and canopy position were the most significant factors controlling the spatiotemporal dynamics in $\Delta R(\text{PAR})$ (Tables 1 and 2). Additionally, a seasonal effect superimposed on the gradual decrease in $\Delta R(\text{PAR})$ with age, presumably due to wax weathering, was also identified. This seasonal effect, detectable in current- and one-year-old needles from the top-canopy, was characterized by lowest values in the winter and highest values in the summer (Fig. 3a). In order to study simultaneously the effect of seasonality and needle age in $\Delta R(\text{PAR})$, we conducted a two-way ANOVA (Table 2). The analysis revealed that seasonality was indeed a significant factor behind the temporal dynamics in $\Delta R(\text{PAR})$. We suggest that the dynamics in wax reflectance found in this study could represent a new photoprotective mechanism operating at the seasonal scale as it modulates PAR absorption over time.

Unlike deciduous plants, overwintering evergreen plants do not shed their leaves annually but retain them for several years. For example,

in Scots pine from boreal and sub-boreal regions as old as eight-year-old needles can be found (e.g. Bäck *et al.* 1994). Thus, leaves of evergreen plants need to cope with the variation in environmental conditions, typical for the northern and alpine regions. In order to ensure the integrity of the photosynthetic apparatus, Scots pine needles undergo drastic seasonal structural and molecular changes at the photosystem level. Some of the changes concern restructuring and changes in size of photon-capturing antennae, and some are related to modifications in the quantity of chlorophylls, non-photosynthetic pigments, photosystem II light harvesting complexes and reaction centres (see Öquist and Huner 2003 for more details). Apart from the adjustments in light absorption, evergreen leaves also alter the energy partitioning at the photosystem level modifying the contribution of dynamic and sustainable thermal dissipation processes that are strongly regulated by light and temperature (Adams and Demmig-Adams 1994, Porcar-Castell *et al.* 2008, Porcar-Castell 2011). All these changes are meant to balance the partitioning of energy at the photosystem level between photochemical and non-photochemical processes, so the excitation pressure at the photosystem II and damage to reaction centres can be reduced. Nevertheless, episodes of excessive light that boreal evergreens experience during the spring months may upset the energy balance as the high irradiance favours light absorption, whilst low temperatures constrain the photosynthetic carbon reactions (Ensminger *et al.* 2004, Porcar-Castell *et al.* 2008, Porcar-Castell 2011).

Apart from the effect that seasonal changes in the photosynthetic apparatus have on the absorption of light, the increase in the reflectance of the epicuticular waxes, $\Delta R(\text{PAR})$, detected from winter towards early summer (Fig. 3a), is consistent with an increased protective role of epicuticular waxes to reduce the excitation pressure and photodamage during cold and bright days in the spring months when the photosystems are subjected to higher levels of excess light than during the relatively dark winter days. In fact, a possible photoprotective role of epicuticular waxes has already been suggested from the comparison between glaucous and non-glaucous leaves of the same plant specimens

(Mohammadian *et al.* 2007), and by comparison of needles of different conifer species (Clark and Lister 1975). These studies, however, were conducted only during a certain point in time. Yet, our study suggests that epicuticular waxes could have a photoprotective role also at the seasonal scale. The fact that the seasonal differences in $\Delta R(\text{PAR})$ disappeared in older than one-year-old needles from the top-canopy might be explained by the low signal in the measurements. In fact, annual mean $\Delta R(\text{PAR})$ from any of the needle ages from the low canopy were half smaller than the $\Delta R(\text{PAR})$ from the current-year needles from the top-canopy. Although we tried to minimize the manipulation of needles during the measurements, removing part of the epicuticular waxes when arranging the needles in the clips was unavoidable, adding a certain degree of measurement noise to the results and a bias towards the underestimation of $\Delta R(\text{PAR})$.

There is ample evidence supporting the decrease in the amount of epicuticular waxes due to wax weathering. Natural environmental factors such as wind (Van Gardingen *et al.* 1991), wind-carried dust and sea salt residues (Turunen and Huttunen 1996), rain (Baker and Hunt 1986, Percy and Baker 1990) or snow and ice formation on needle surfaces contribute to eroding and fracturing of the epicuticular wax crystallites. Furthermore, air pollution (Cape 1986, Turunen and Huttunen 1990), ozone (Günthardt-Goerg and Keller 1987) or acid rain and fog (Percy *et al.* 1992) has been shown to enhance natural wax weathering. However, pollution levels at our study site are rather low (Kulmala *et al.* 2000) and thus their effects are probably minor. It has also been suggested, on the basis of scanning electron microscopy, that part of what has been described as weathered waxes in aged needles might actually be deliquescent hygroscopic particles, which have similar amorphous appearance (Burkhardt 2010). Still, as wax weathering has also been related to the shortening and thickening of the wax crystallites into flatten amorphous structures (Cape 1986, Crossley and Fowler 1986), the weathered waxes could reflect less light and explain the decrease in $\Delta R(\text{PAR})$ found with needle ageing. In fact, reflectance of intact needles, $R_w(\lambda)$, progressively declined from the newest to the oldest needles as a conse-

quence of wax weathering (Fig. 1). As a result, lower $R_w(\lambda)$ profiles produce lower epicuticular wax reflectance profiles, $\Delta R(\lambda)$, and $\Delta R(\text{PAR})$, that might have impaired the detection of any seasonal trend under the superimposed effect of needle ageing.

Differences in the intensity of some environmental factors such as wind and rain within the canopy could also alter wax weathering rates, thus explaining the differences in $\Delta R(\text{PAR})$ between both canopy positions (Figs. 1 and 3). Apart from wax weathering effects, these differences may also reflect distinctive micro-environmental conditions within the canopy. The air temperature during wax synthesis has been shown to affect the development of epicuticular waxes of the same plant species into different structures (Baker 1974), and thus their PAR reflectance properties. In fact, air temperature has been shown to affect the glaucousness of juvenile leaves of *Eucalyptus bicostata* (Cameron 1970). However, we did not find variations in air temperature between the canopy positions (Fig. 2a), and thus the observed differences in $\Delta R(\text{PAR})$ within the canopy of Scots pine are unlikely to be caused by temperature. In contrast, PAR within the canopy revealed a strong gradient (Fig. 2b), reflecting differences in the light environments in each of the canopy positions, and the associated differences in the photosynthetic performance and optical properties between shaded needles and those in direct sunlight.

Concluding remarks

The results of our study demonstrate that the optical properties of epicuticular waxes undergo a complex temporal pattern during the lifespan of the needle. We found a seasonal pattern of variation superimposed on that of the natural effect of wax weathering with age, which has important implications. First, the spatiotemporal dynamics in wax $\Delta R(\text{PAR})$ identified here should be considered in studies that implicitly assume constant light absorption, and particularly whenever different leaf age classes, canopy positions or seasons are compared (e.g. comparing electron transport rates obtained fluoro-

metrically or light response curves and light use efficiencies obtained via gas exchange). Furthermore, although absolute values of $\Delta R(\text{PAR})$ found in this study for Scots pine as a model species were not very large (up to 3%), the effect and significance of the photoprotective role proposed here might become more important in species having higher amounts of epicuticular waxes. Elucidating the functional role of these dynamics will require further studies at higher temporal resolutions.

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