Dissertationes Forestales 247

Leaf optical properties and dynamics of photosynthetic activity

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Academic Dissertation
To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium ls5 of Metsätieteiden Talo (Latokartanonkaari 7), on March 9th, 2018 at 12 o'clock noon.

Title of dissertation: Leaf optical properties and dynamics of photosynthetic activity

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Dissertationes Forestales 247

http://dx.doi.org/10.14214/df.247

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ISSN 1795-7389 (online)
ISBN 978-951-651-584-0 (pdf)

ISSN 2323-9220 (print)
ISBN 978-951-651-585-7 (paperback)

Publishers:
Finnish Society of Forest Science
Faculty of Agriculture and Forestry at the University of Helsinki
School of Forest Sciences at the University of Eastern Finland

Editorial Office:
Finnish Society of Forest Science
Viikinkaari 6, 00790 Helsinki
http://www.dissertationesforestales.fi

Olascoaga B. (2018). Leaf optical properties and dynamics of photosynthetic activity. Dissertationes Forestales 247. 55 p. Available at https://doi.org/10.14214/df.247

ABSTRACT

Photosynthesis requires a balance between its light-dependent and light-independent reactions so that the energy input through photochemistry matches its consumption. Biochemical and physiological processes help to achieve this balance, as certain processes regulate the activity of light-dependent photochemical reactions, whilst others regulate the activity of temperature-dependent biochemical reactions. Biochemical and physiological processes also modulate the absorbed energy available for photosynthesis by diverting a fraction into non-photochemical pathways that dissipate energy as heat and fluorescence. Interestingly, certain biochemical and physiological processes behind the dynamics of photosynthesis correlate with leaf optical properties (LOPs), which represent an approach to characterising the dynamics of photosynthesis. Yet, how solid is our knowledge concerning the biochemical and physiological processes influencing LOPs, and how accurately do LOPs and the biochemical and physiological processes behind photosynthetic dynamics correlate when investigated across various spatio-temporal scales? This thesis investigated whether reflectance-based and fluorescence-based LOPs adequately correlate with the biochemical and physiological processes behind photosynthetic dynamics, and whether their correlations hold true at various spatio-temporal scales.

This thesis demonstrates the validity of reflectance-based and fluorescence-based LOPs as optical proxies for investigating the dynamics of photosynthesis. However, it also identifies sources of variability that cause the correlations between photosynthesis and LOPs to break down. This thesis classifies the sources of variability in terms of methodological (i.e. over-simplification and technical/instrumental constraints) and spatio-temporal limitations. The over-simplification of processes behind the dynamics of photosynthesis and LOPs was addressed by studying the absorption of photosynthetically active radiation (PAR) by conifer needles. PAR absorption is generally considered to be chlorophyll concentration-dependent, yet this thesis shows it to be additionally modulated by the effect that waxes have on needle PAR reflectance. Due to the difficulties of directly measuring needle PAR absorption, PAR reflectance was used as a proxy of PAR absorption. To solve this technical/instrumental constraint, this thesis presents a new methodology that facilitates the direct estimation of PAR absorption. This thesis also demonstrates that certain LOPs appear to be insensitive to detecting the dynamics of certain biochemical and physiological processes over time. This was true for the photochemical reflectance index (PRI), which failed to detect zeaxanthin-independent processes behind the thermal dissipation of the absorbed PAR. Lastly, this thesis shows that LOPs can also be influenced by leaf morphology, which could affect the optically-based monitoring of larger-than-leaf scales. Despite the caveats highlighted in this thesis, the potential to monitor the dynamics of photosynthetic activity by optical means is unquestionable, and the results presented here can contribute to reducing uncertainty in the characterisation of photosynthesis by optical means at varying spatio-temporal scales.

Keywords: absorption, fluorescence, non-photochemical quenching (NPQ), photochemical reflectance index (PRI), photochemical yield (ϕ_P), reflectance

Acknowledgements

Several people have, directly or indirectly, helped me accomplish this thesis. Even before starting my studies in Finland, Beatriz Fernández-Marín, José Ignacio García-Plazaola and Raquel Esteban provided me an initial contact with science when I was still a student at the University of the Basque Country. My fascination with plant physiology and optics began while already in Leioa.

Beginning my doctoral studies at the University of Helsinki would not had been possible without all the help Eero Nikinmaa gave me. I would like to thank the Basque Government and the Academy of Finland for the financial support provided for conducting my studies, and to all co-authors whose contributions enabled the publication of these studies. Achieving my research goals would have been simply impossible without all the knowledge, experience and support that my supervisors Albert Porcar-Castell, Eija Juurola, Jaana Bäck and Jon Atherton offered me. I would like to give a special thanks to Albert Porcar-Castell for accepting to be my main supervisor, and for mentoring me in the optics behind photosynthesis. Your support and patience have been invaluable to me. El camí no ha estat sempre fàcil, però espero que hagis gaudit el viatge tant com jo.

I would also like to thank all the people belonging to the Ecosystem Processes Group for making me feel part of something larger, something that what my doctoral studies, with their precise and detailed research questions, might fail to show. Kokonaisuus on suurempi kuin osien summa. Kiitos kaikille. The friends that I have made beyond this group have always been supportive and helped me, making my stay in Finland memorable. Thank you Anne, Clara, Renato, Jenny and Windi. I would also like to thank all the Biolokos back from my University period and the members of Portuarraken Asoziayua from back home. You have always supported me. It goes without mention to thank my mother Amaya and my brother Igor, for all the support and understanding during these years. I would also like to give a special thanks to Víctor. Porque todo esto habría sido muy distinto sin tu apoyo. Muchas gracias.

Amaitzeko, doktoretza-tesi hau nire Atta eta Amona Xalu zirenei eskaini nahiko nieke, guzti honen hasieraren lekuko izan, baina tamalez bukaera ikusi izan ez dutenei.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following publications, which are referred to in the text by their Roman numerals. All the articles are reprinted with the kind permission of the publishers.

I. Olascoaga B., Juurola E., Pinho P., Lukeš P., Halonen L., Nikinmaa E., Bäck J., Porcar-Castell A. (2014). Seasonal variation in the reflectance of photosynthetically active radiation from epicuticular waxes of Scots pine (*Pinus sylvestris*) needles. Boreal Environment Research 19 (suppl. B): 132-141. http://hdl.handle.net/10138/165185

II. Olascoaga B., Mac Arthur A., Atherton J., Porcar-Castell A. (2016). A comparison of methods to estimate photosynthetic light absorption in leaves with contrasting morphology. Tree Physiology 36(3): 368-379. https://dx.doi.org/10.1093/treephys/tpv133

III. Porcar-Castell A., García-Plazaola J.I., Nichol C.J., Kolari P., Olascoaga B., Kuusinen N., Fernández-Marín B., Pulkkinen M., Juurola E., Nikinmaa E. (2012). Physiology of the seasonal relationship between the photochemical reflectance index and photosynthetic light use efficiency. Oecologia 170(2): 313-323. https://dx.doi.org/10.1007/s00442-012-2317-9

IV. Atherton J., Olascoaga B., Alonso L., Porcar-Castell A. (2017). Spatial variation of leaf optical properties in a boreal forest is influenced by species and light environment. Frontiers in Plant Science 309(8):1-14. https://dx.doi.org/10.3389/fpls.2017.00309

Author's contribution:

Beñat Olascoaga was the author of the thesis summary, and the main author of **Studies I** and **II**. Olascoaga collected the field materials used in **Studies I** and **II**, and processed and analysed all the data used in **Studies I** and **II**. In **Study III**, Olascoaga helped process the samples and participated in commenting on the article. In **Study IV**, Olascoaga set up and measured the leaf absorption of radiation as well as summarised the survey data. Olascoaga also participated in commenting on the article. All co-authors participated in discussing and commenting on the articles.

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SYMBOLS AND ABBREVIATIONS

A: Antheraxanthin
A_B: Bidirectional absorption
A_{BLACK}: Hemispherical absorption of a black paint for the PAR region
ADP: Adenosin diphosphate
A_H: Hemispherical absorption factor
A_T: Total absorption
ATP: Adenosin trisphosphate
ETR: Electron transport rate
F'_m: Maximal chlorophyll *a* fluorescence measured in an illuminated leaf after a saturating light pulse
F: Chlorophyll *a* fluorescence measured in an illuminated leaf at any point in time
F_m: Maximal chlorophyll *a* fluorescence measured in a dark-adapted leaf after a saturating light pulse
F_{mR}: Reference F_m
F_o: Minimal chlorophyll *a* fluorescence measured in a dark-adapted leaf after a saturating light pulse
FRET: Förster resonance energy transfer
G_F: Gap fraction
GNDVI: Green Normalised Difference Vegetation Index
GPP: Gross primary production
LOP: Leaf optical property
LUE: Light use efficiency
I_B: Photon flux density of the blackened sample inside the integrating sphere for A_T estimation
I_{S_RH}: Photon flux density of the leaf for R_H estimation
I_S: Photon flux density of the sample inside the integrating sphere for A_T estimation
I_{S_RB}: Photon flux density of the leaf sample for R_B estimation
I_{S_TH}: Photon flux density of the sample for T_H estimation
I_{STR_RH}: Photon flux density of a light trap for R_H estimation
I_{STR_TH}: Photon flux density of a light trap for T_H estimation
I_W: Photon flux density of an empty integrating sphere for A_T estimation
I_{W_RB}: Photon flux density of a white reference for R_B estimation
I_{W_RH}: Photon flux density of a white reference for R_H estimation
I_{W_TH}: Photon flux density of a white reference for T_H estimation
$NADP^+$: Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)
NDVI: Normalised difference vegetation index
NIR: Near-infrared
NPP: Net primary production
NPQ: Non-photochemical quenching of the chlorophyll *a* fluorescence signal
P_{680}: Chlorophyll of PSII with absorption peak at 680 nm
P_{700}: Chlorophyll of PSI with absorption peak at 700 nm
PAR: Photosynthetically Active Radiation
PRI: Photochemical Reflectance Index
PSI: Photosystem I
PSII: Photosystem II

PQ: Photochemical quenching of the chlorophyll *a* fluorescence signal
RC: Reaction centre
R_n: Reflectance at 'n' nm wavelength
R_W: Reflectance of integrating sphere walls
R_B: Bidirectional reflectance factor
R_H: Hemispherical reflectance factor
R_{SP}: Reflectance of a white reference panel
T_H: Hemispherical transmittance factor
UAV: Unmanned aerial vehicle
V: Violaxanthin
Z: Zeaxanthin
ΔpH: Trans-thylakoid membrane proton gradient
ϕ_P: Photochemical yield

1. INTRODUCTION

1.1 Photosynthesis in the boreal forest

The optical properties of plants are a source of information. For example, visible radiation reflected from fruits and flowers influences their conspicuousness to pollinators and seed dispersers (Renoult et al. 2014), whilst ultraviolet radiation reflectance from the petals affects the conspicuousness of flowers to pollinators (Koski and Ashman 2014). Visible and infrared radiation detected by plants contains information of the surrounding environment and the presence of vegetation (Ballaré and Pierik 2017). Biochemical and physiological processes influencing plant photosynthetic activity also influence leaf optical properties (LOPs) over time, which can then be exploited to monitor photosynthesis by optical means. This thesis deepens the potential of LOPs (in terms of reflected and emitted visible radiation) as a source of information to describe the dynamics of biochemical and physiological processes influencing photosynthesis under various scales of space and time.

Photosynthesis is the metabolic process conducted by photoautotrophic organisms, such as plants, through which carbon dioxide (CO_2) in the atmosphere (in the case of terrestrial plants) or water (in the case of aquatic plants) is turned into glucose and other sugars in a process that uses visible radiation as its energy source. Thus, photosynthesis has ecological implications for the carbon (C) cycle, as it influences atmospheric CO_2 concentrations. The net amount of carbon fixed through photosynthesis, also known as net primary production (NPP), is estimated to reach ~100–500 Pg C annually at a global scale, ~50% of which originates in terrestrial biomes (Falkowski and Raven 2007; Friend et al. 2009, Antal et al. 2013). Nevertheless, photosynthetic rates and NPP differ between various biomes.

This thesis concentrates on the dynamics of photosynthesis and the associated LOPs in trees of the Finnish boreal forest. At the global scale, ~30% of the terrestrial area is covered by forests (FAO 2010). Approximately 50% of the forested area belongs to the boreal type (Brandt et al. 2013). The boreal forest is thus an extensive biome with a significant impact on terrestrial C accumulation and atmospheric CO_2 dynamics. The C stock accumulated within all the forests of the planet is estimated at ~861±66 Pg C, ~272±23 Pg C of which are stored in the boreal forest (Pan et al. 2011). Yet, photosynthetic rates and NPP not only differ between biome types but also over time, because photosynthesis must constantly adjust in response to climatic and physiological constraints.

The boreal zone is delimited between latitudes 50° and 70° N (Baldocchi et al. 2000; Johnson and Miyanishi 2012), and it is characterised by large temperature and irradiance variations during the year, influencing the physiology of the plant and its capacity to conduct photosynthesis. In terms of temperature, certain boreal regions can experience temperatures up to 30 °C during summer, whilst temperatures in winter can drop to -70 °C (Baldocchi et al. 2000). In terms of irradiance, certain regions beyond the Arctic Circle experience periods during winter when the sun never rises (i.e. polar night) and periods in summer when the sun never sets (i.e. midnight sun). These large temperature and irradiance variations cause short growing seasons in the boreal forest, with less than 120 days in certain regions (Baldocchi et al. 2000; Brandt 2009).

The boreal zone is also less diverse in tree species than the temperate and tropical zones (Gauthier et al. 2015). However, the perennial genera of pine (*Pinus*), spruce (*Abies*) and fir

(*Picea*) are often present along with deciduous genera such as larch (*Larix*), birch (*Betula*), aspen (*Populus*), willow (*Salix*) and alder (*Alnus*) (Soja et al. 2007). The photosynthetic activity of deciduous trees, which are mainly broadleaved species, is limited to the growing season from leaf budburst during spring to leaf shed during autumn. Nevertheless, the leaves of perennial trees, which are mainly needle-like, are not shed after the growing season, but remain in the canopy for many years (Bäck et al. 1994; Dengel et al. 2013). Due to this foliar habit, perennial leaves must perform drastic biochemical and physiological adjustments over the year to cope with the dramatic differences in temperature and irradiance typical to the boreal zone. They must also achieve a balance between the light-dependent and light-independent reactions of photosynthesis over time.

Certain biochemical and physiological processes affecting the light-dependent reactions of photosynthesis influence LOPs, which can be optically detected. A theoretical framework therefore exists that supports a correlation between LOPs and the light-dependent reactions of photosynthesis. As plants tend to reach a photostatic balance between the energy input through photochemistry and the energy consumption by the light-independent reactions of photosynthesis, LOPs can be exploited as an approach to study photosynthetic activity. But how well are LOPs related to the spatio-temporal dynamics of photosynthesis? The next sections of this thesis introduce the processes behind the dynamics of photosynthetic activity and LOPs along with the challenges and assumptions behind their correlation, identifying methodological and spatio-temporal limitations in the relationship between LOPs and photosynthetic activity.

1.2 Pigments and photosystems

Photosynthesis occurs within the chloroplasts of cells (Fig. 1). Chloroplasts are organelles ~5–7 µm in length and ~2.5 µm in width (Antal et al. 2013), which contain a network of specialised membranes (i.e. thylakoids) embedded in the chloroplastic cytoplasm (i.e. stroma). The components responsible for the various photosynthetic reactions are located on the thylakoids. Protein-pigment complexes known as photosystems are one of the main components. The pigments associated with photosystems are capable of absorbing photons along the visible region of the electromagnetic spectrum. This region is also the region of photosynthetically active radiation (PAR, 400–700 nm). PAR is thus fundamental for the photosynthetic process, because the energy associated with the absorbed PAR can ultimately be used for CO_2 assimilation. However, not all radiation in the PAR region can be evenly absorbed by the pigments, as various pigment types have different absorption spectra.

Chlorophylls are one of the most abundant pigments on the photosystems. These magnesium-containing chlorin rings have absorption maxima in the blue and red regions, and an absorption minimum in the green region of the PAR region. Higher plants have two types of chlorophylls, i.e. chlorophyll *a* and chlorophyll *b*, which differ in their functional groups and hence in their absorption spectra. The absorption maxima of chlorophyll *a* are approximately 410, 430 and 660 nm, while chlorophyll *b* has absorption maxima around 430, 460 and 640 nm (Papageorgiou 2004; Antal et al. 2013). The absorption of irradiance within the *ca*. 400–500 nm region of PAR is strong, not only due to chlorophylls, but also due to the existence of carotenoids (Zur et al. 2000; Kume 2017). Carotenoids are the remaining accessory pigments found in the photosystem and they constitute a group of

tetraterpene-derived pigments consisting of the carotenes and their oxygenated derivatives, i.e. the xanthophylls (Antal et al. 2013).

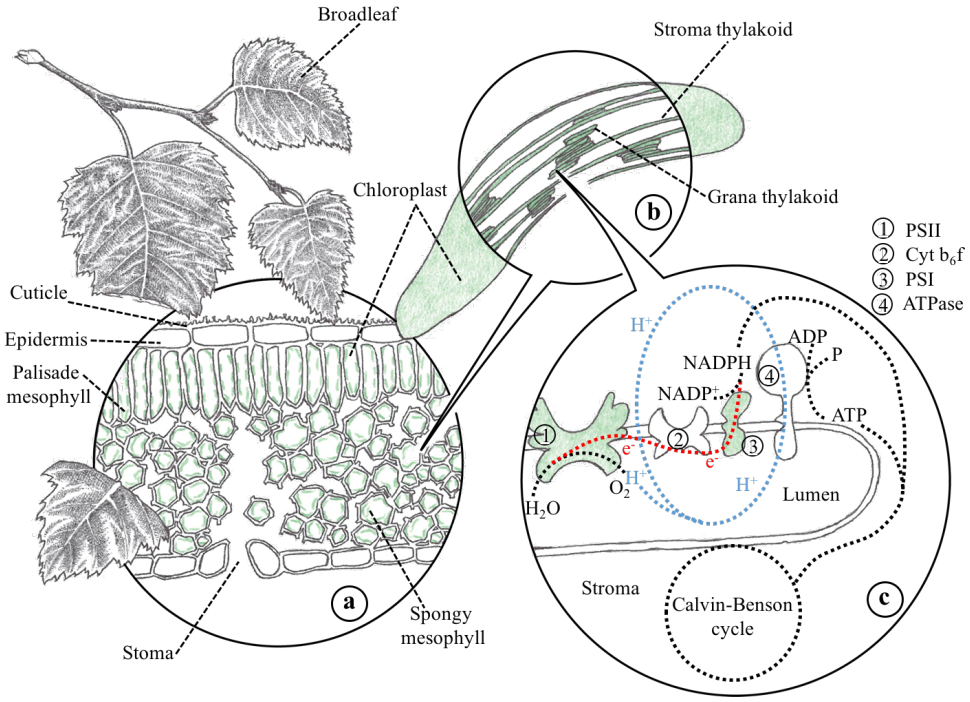

Figure 1. Illustration of **a)** chloroplast location within the palisade and spongy mesophylls, shown in the cross section of a broadleaf. **b)** Cross section of a chloroplast, showing thylakoid dispositions with the grana and stroma thylakoids. **c)** Cross section of a thylakoid, exhibiting the arrangement of photosystem I (PSI) and photosystem II (PSII), and the Cytochrome b_6f complex, constituent of the electron transport chain of the light-dependent reactions of photosynthesis. The Calvin-Benson cycle represents the light-independent reactions of photosynthesis, and ATP synthase (ATPase) is involved in the photophosphorylation process. The red line shows the route of the electrons (e^-) from the splitting of H_2O to the reduction of $NADP^+$ into NADPH. The blue line shows the route of protons (H^+) released from the splitting of H_2O and H^+ pumping into the lumen, used for the phosphorylative synthesis of ATP from ADP and inorganic phosphorus (P).

Plants have two types of photosystems: photosystem I (PSI) and photosystem II (PSII). Protein-pigment complexes in PSI and PSII differ, and the chlorophylls associated with the two types of photosystems therefore absorb at slightly different spectral ranges. The photosystems collect radiation that can potentially be used to conduct photosynthesis. Nevertheless, they are only one of the many components that uphold the entire photosynthetic process, which consists of various light-dependent and light-independent reactions. These reactions will be introduced in Section 4, but it is still necessary to clarify

the various physiological processes in which the absorbed radiation can be partitioned once collected at the photosystems.

1.3 Energy partitioning at the photosystems

A chlorophyll that absorbs a photon reaches an excited state because the energy associated with the photon (also known as excitation energy or exciton) alters its electronic state. This excitation energy causes one of the chlorophyll's electrons to migrate to a more energetic orbital within the chlorophyll molecule from the formerly occupied ground orbital (S_0). When the electron is raised beyond the S_1 orbital, e.g. due to the absorption of a more-energetic blue photon instead of a red photon (Fig. 2), part of its energy is constitutively dissipated as heat by internal conversion, until it is eventually lowered to the S_1 orbital. Additional thermal decay lowers the electron to the lowest sub-orbital within the S_1 orbital. (Valeur 2001; Porcar-Castell et al. 2014).

At this orbital, the excitation energy can be partitioned into different pathways, as it can now be: i) released as heat, ii) emitted as a photon (i.e. chlorophyll *a* fluorescence emission) or iii) transferred to an adjacent chlorophyll, predominantly through Förster resonance energy transfer (FRET) (Şener and Schulten 2005; Novoderezhkin and van Grondelle 2010). FRET causes the excited chlorophyll to relax, and an adjacent chlorophyll to reach its excited state. Due to the many FRET events within the light-harvesting complexes of the photosystems, the exciton finally reaches the reaction centre (RC) of the photosystem. At the photosystem RC, a special chlorophyll conducts a charge separation that transforms the excitation energy into chemical energy (Krause and Weis 1991), which can finally be used to drive photosynthesis. Nevertheless, the partitioning of the excitation energy at the photosystems reveals that photosynthesis can only occur when the excitation energy is diverted to the photochemical pathway through FRET. However, the photochemical pathway is in competition with the pathways that thermally and optically release the absorbed excitation energy. All PAR absorbed by leaves is not necessarily used to drive photosynthesis. The competition between the pathways for the excitation energy is dynamic, and will therefore vary over time under the influence of biotic and abiotic factors.

Interestingly, as one of the pathways involved in the partitioning of the excitation energy is optically detectable (i.e. chlorophyll *a* fluorescence), changes in energy partitioning and hence the dynamics of the photosynthetic activity can be monitored by means of chlorophyll *a* fluorescence detection. Additionally, optical features of the radiation reflected by plants can also be related to the thermal dissipation of the excitation energy, and hence to the dynamics of photosynthetic activity. But what are the biochemical and physiological processes behind the dynamics of photosynthetic activity and how is it possible to monitor them by optical means?

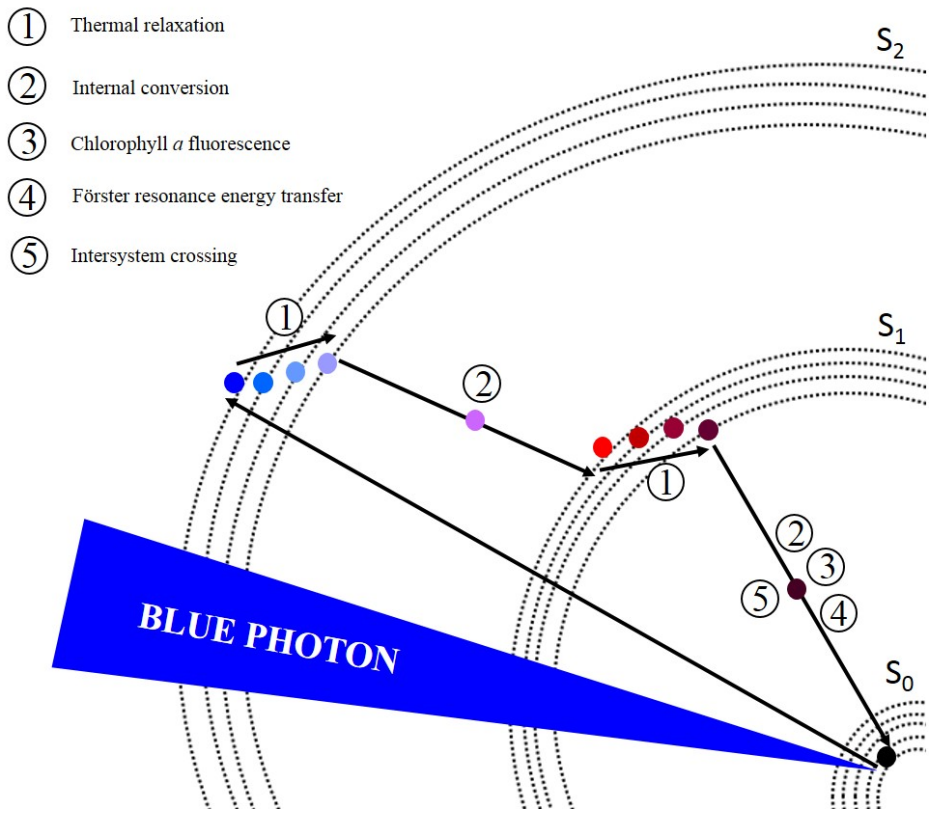

Figure 2. Representation of the Jablonski diagram showing the excitation and de-excitation pathways of an electron. When chlorophyll *a* absorbs a photon (e.g. a blue photon), it reaches an excited state, as one of its electrons migrates to a more energetic orbital. Part of the absorbed energy is released as heat within the orbital due to thermal relaxation, and internal conversions allow it to reach lower electronic orbitals by thermal dissipation. From S_1 to S_0 ground orbital, the absorbed energy is not only thermally released, but can also be dissipated by Förster resonance energy transfer (FRET) to a nearby chlorophyll molecule, or by the emission of the absorbed energy as a photon of chlorophyll *a* fluorescence. An additional process, i.e. intersystem crossing, may produce a triplet-state chlorophyll. The solid circles represent electrons, with colours ranging from blue to black illustrating a scale from the highest to the lowest energy levels. Each of the dotted lines represents sub-orbitals within the S_2, S_1 and S_0 orbitals of the molecule.

1.4 Light-dependent and light-independent reactions of photosynthesis

As previously stated, the photosynthetic process comprises two sets of reactions that work in series to successfully transform CO_2 and radiation into sugars (Hüner et al. 1996; Ensminger et al. 2004). One of these reaction sets is constituted by light-dependent reactions, whilst the remaining reaction set forms the light-independent reactions of photosynthesis (Fig. 1c).

Light-dependent reactions of photosynthesis occur in the thylakoid membrane, where the excitation energy absorbed by PSII chlorophylls and accessory pigments is eventually transferred to a special chlorophyll, i.e. P_{680}, located at the PSII RC. The absorbed excitation energy is thus transformed into chemical energy, as the excited P_{680} is able to release an electron to pheophytin (Pheo). Pheo then transfers the electron to the primary quinone acceptor, i.e. quinone A (Q_A), initiating the electron transport from PSII to PSI through the Cyt b_6f complex (Fig. 1c) (Krause and Weis 1991; Cardona et al. 2012; Porcar-Castell et al. 2014). Electrons transported to the Cyt b_6f complex cause the pumping of protons into the lumen (i.e. the cytosolic environment within the thylakoids), which become acidified during the process. The oxidised P_{680} eventually receives a new electron from an H_2O molecule that is split into molecular oxygen (O_2), electrons and protons by the PSII oxygen-evolving complex (OEC) (Antal et al. 2013; Gupta et al. 2015).

The excitation energy absorbed by PSI chlorophylls and accessory pigments simultaneously reaches the special chlorophyll at the PSI RC known as P_{700}. P_{700} transfers an electron to ferredoxin (Fd) via sulfur-iron complexes. An electron from the reduced Fd is then transferred to $NADP^+$ through the enzyme ferredoxin-$NADP^+$ reductase (FNR), transforming $NADP^+$ into NADPH (Joliot and Johnson 2011). This provides the reducing power to be used in the light-independent reactions of photosynthesis. The oxidised P_{700} eventually recovers its electronic state with the help of plastocyanin (Pc), which is responsible for transporting electrons from the Cyt b_6f complex to PSI.

Concomitantly, the protons that were pumped into the lumen through the Cyt b_6f complex and those made available from the splitting of H_2O by OEC are released back to the stroma through an ATPase. The ATPase uses proton-motive energy to bind ADP and inorganic phosphorus as ATP via photophosphorylation (Jagendorf 2002). Thus, the photosynthetic process acquires the NADPH and ATP molecules that are used in the light-independent reactions of photosynthesis. These molecules are acquired through reactions involved in the light-dependent reactions of photosynthesis that utilise PAR and H_2O (Antal et al. 2013). During the light-independent reactions of photosynthesis, CO_2 is sequestered by the enzyme ribulose-1,5-bisphospate carboxylase/oxygenase (rubisco) located at the stromal side of the thylakoids. Rubisco is an essential enzyme of the Calvin-Benson cycle, in which the recently captured atmospheric CO_2 is reduced into sugars. (Cen and Sage 2005).

Light-dependent reactions of photosynthesis are mainly temperature-insensitive photochemical processes strongly affected by PAR quantity and quality. However, light-independent reactions of photosynthesis are largely independent of the light conditions, but are temperature-sensitive biochemical processes (Öquist and Hüner 2003). Thus, the whole photosynthetic process needs fine-tuning between its light-dependent- and light-independent reactions.

1.5 Photostasis: the balance between light-dependent and light-independent reactions of photosynthesis

Imbalances between the two photosynthetic reactions (i.e. light-independent and light-dependent reactions) reduce photosynthetic performance and can compromise the integrity of the photochemical apparatus and ultimately the integrity of the cell. The duration of the excitation energy within the photosystem lengthens when the amount of absorbed excitation energy is excessive and cannot be promptly dissipated because e.g. low temperatures

constrain the optimal consumption rates of NADPH and ATP. Imbalances between the rates of energy absorption and its consumption alter the redox state of the electron transport chain components, measurable in term of PSII excitation pressure (Hüner et al. 1996; Hüner et al. 1998), increasing the possibility of photo-oxidative damage on over-excited photosystems due to the formation of reactive oxygen species (ROS). Excited chlorophylls that uphold the excitation energy for a lengthy time can turn into triplet chlorophylls ($^3Chl^*$, Fig. 2) (Krieger-Liszkay 2004), which can ultimately convert O_2 into singlet oxygen ($^1O_2^*$) (Jahns and Holzwarth 2012). This can damage components of the photosynthetic apparatus such as the PSII RC D1 protein. Even electrons directly donated to O_2 can produce ROS by generating a superoxide radical (O_2^-), which in turn can be transformed into hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$).

To prevent potential damages derived from energetic imbalances, plants have evolved mechanisms to achieve photostatis (Hüner et al. 2003; Öquist and Hüner 2003; Ensminger et al. 2006), i.e. a balance between the energy input through photochemistry and its consumption. Photostasis can be achieved through diverse biochemical, morphological and physiological mechanisms operating at various parts of the leaf, from the leaf surface to the photosystems. Additionally, some of these mechanisms function during short-term periods (e.g. seconds to hours), whilst others are more effective for longer periods, i.e. from seasonal to annual scales.

Short-term mechanisms in plants have evolved to modify the leaf area capable of intercepting radiation, thus regulating the quantity of irradiance to absorb. For example, certain plants alter the leaf orientation angle with respect to the direction of irradiance and thus, during episodes of excessive radiation (e.g. midday on sunny days), their leaves gain a more vertical orientation to reduce the leaf area exposed to irradiance (Muraoka et al. 1998; Joesting et al. 2016). For similar purposes, certain plants fold their leaf margins during episodes of excessive irradiance (Huang et al. 2012).

Once a leaf intercepts radiation, the quantity of irradiance penetrating the leaf can still be reduced at the leaf surface by means of epicuticular waxes (Shepherd and Griffiths 2006; Esteban et al. 2014), trichomes (Holmes and Keiller 2002), salt deposits (Esteban et al. 2013) and other epidermal structures (Vogelmann 1993). Epicuticular waxes constitute complex mixtures of C_{20}–C_{40} aliphatic hydrocarbons and their derivatives (Koch and Ensikat 2008; Domínguez et al. 2011), which are located on the cuticle surface, and in most cases protrude hundreds of nanometres to a few micrometres from the aggregates, forming wax crystals of diverse shape (Barthlott et al. 1998; Koch and Ensikat 2008). Epicuticular wax crystals can reflect radiation that can potentially be used for photosynthesis. Usually leaves reflect 5–10% of the incident visible radiation (Vogelmann 1993), but denser wax coverings (i.e. glaucous leaves) can increase leaf reflectance (Holmes and Keiller 2002). Regeneration of epicuticular waxes appears to be species-dependent, some species being unable to regenerate them while other species regenerate variable amounts of wax during various leaf developmental stages (Neinhuis et al. 2001). However, the role played by epidermal structures, such as epicuticular waxes, in the dynamics of photosynthetic activity has only been studied for certain biotic and abiotic stresses (e.g. water stress and air pollution) along with leaf early developmental stages, and their optical properties under various spatial and temporal scales still remain poorly characterised.

Once radiation penetrates the leaf, rapid chloroplast movements act as a short-term mechanism modifying the quantity of irradiance potentially available for photosynthesis. This is done by modulating the intercepting area of absorbable visible radiation at the cellular level (Kasahara et al. 2002; Königer and Bollinger 2012). Even at the photosystem

level within the chloroplasts, state transitions can minimise the excitation pressure on the photochemical apparatus by balancing the proportion of visible radiation absorbable by PSI and PSII through temporal displacements of light-harvesting complexes between both photosystem types (Wollman 2001; Tikkanen et al. 2011). A fraction of the energy absorbed at the PSII light-harvesting complexes can also be thermally dissipated instead of being used in photosynthesis (Porcar-Castell 2011; Porcar-Castell et al. 2014). Thermal dissipation of the absorbed energy occurs when imbalances between the light-dependent and light-independent reactions alter the electron transport rate between both photosystems, increasing the trans-thylakoid membrane proton gradient (ΔpH). The ΔpH increase is sensed by the PsbS protein (Li et al. 2004) and the violaxanthin de-epoxidase enzyme, which transforms the xanthophyll pigment violaxanthin (V) into antheraxanthin (A) and zeaxanthin (Z) (Müller et al. 2001). PsbS protein protonation and V de-epoxidation facilitate the thermal dissipation of the excitation energy from photosystems through a reversible process (Demmig-Adams and Adams 2006). Lower light intensities or dark conditions epoxidise Z and A back to V and de-protonate PsbS proteins, deactivating the thermal dissipation process.

At the seasonal scale, plants also have mechanisms to balance between energy input through photochemistry and its consumption demands. During the lifespan of a leaf, seasonal adjustments occur in the concentrations of chlorophylls (Powles 1984; Niinemets 2010), carotenoids (Han et al. 2003), anthocyanins (Chalker-Scott 1999; Gould 2004), flavonoids (Rozema et al. 1997; Agati et al. 2012) and other pigments that can selectively filter specific wavelengths of the electromagnetic spectrum, and hence modify the quantity of absorbable radiation over time. Plants additionally adjust the number of leaves over time (Hoffmann et al. 2005), which could influence the amount of radiation to be absorbed. However, deciduous species shed their leaves during unfavourable seasons, but evergreen species retain their foliage all year round. Because of their foliar habit evergreen species undergo drastic seasonal changes in their photochemical apparatus between the favourable (i.e. spring and summer) and unfavourable seasons (i.e. autumn and winter), switching from a system highly efficient at harvesting light for photochemistry during the favourable season to a system highly efficient at thermal dissipation during the unfavourable season (Öquist and Hüner 2003). During this time the photosystems are still capable of absorbing radiation, but the low temperatures largely inhibit the enzymatic processes of photosynthesis.

This high efficiency in thermal dissipation occurs because the decrease in photoperiod and temperature that evergreen trees sense in autumn triggers a cold-hardening process that ultimately produces a down-regulation of photosynthesis. It also entails organisational changes in photosystems along with its protein and pigment concentrations (Ottander et al. 1995; Ensminger et al. 2004; Zarter et al. 2006). A reduction occurs in antenna size and in the number of chlorophylls (Vogg et al. 1998), allowing the amount of absorbable radiation to decrease in comparison to levels found during the favourable seasons. A reduction in the number of PSII RCs also occurs, as detected by means of D1 protein degradation (Savitch et al. 2002). Additionally, PsbS protein concentration increases (Öquist and Hüner 2003) and the de-epoxidation state of the VAZ xanthophyll cycle pigments increases. This enhances a sustained form of thermal dissipation that is independent of the trans-thylakoid ΔpH (Öquist and Hüner 2003; Demmig-Adams and Adams 2006; Verhoeven 2014), therefor does not relax in the short term during low light intensities or complete darkness. Lastly, the activation of energy-consuming pathways alternative to the linear electron transport of photosynthesis, e.g. cyclic electron pathways (Munekage et al. 2004; Rumeau

et al. 2007) and pseudocyclic electron pathways, such as the Mehler-ascorbate peroxidase pathway (Asada 1999; Ivanov et al. 2002), can also reduce the excitation pressure on the photosystems by temporarily diverting away excessive excitation energy. As an example, the cyclic electron transport around PSI appears to increase in plants acclimated to coldness (Ivanov et al. 2001; Ivanov et al. 2012) and during the last stages of leaf senescence (Kotakis et al. 2014).

Interestingly, temporal changes in LOPs appear to correlate with many of these processes behind the dynamics of photosynthesis, which enables the detection of photosynthetic activity dynamics through optical means. The following section describes the origins of the most commonly used LOPs, and how they correlate with certain biochemical, morphological and physiological processes explained in this section.

1.6 Linking LOPs to photosynthesis

One of the advantages of studying the dynamics of photosynthetic activity through LOPs is that LOPs can be determined through non-destructive and non-invasive optical approaches (e.g. spectroradiometry and fluorometry), which detect the radiation reflected and emitted from the leaf. Optical approaches are also potentially less time-consuming than wet laboratory-based approaches such as pigment extraction and analysis. They also allow monitoring the same leaf sample at any temporal scale from leaf flush to the late leaf senescence stages. Furthermore, LOPs can also be detected for larger spatial scales than the leaf with the help of platforms and airborne (e.g. unmanned aerial vehicles i.e. UAVs) and spaceborne (e.g. satellites) devices, extending the target of study to the shoots, canopies, landscape, etc.

LOPs are classified into two categories: i) those derived from reflected radiation (i.e. reflectance and transmittance) and ii) those derived from emitted radiation (i.e. chlorophyll *a* fluorescence).

1.6.1 LOPs derived from reflected radiation: leaf reflectance and transmittance

Radiation incident on the leaf surface is either absorbed, transmitted or reflected (Fig. 3), depending on the wavelength and on the biochemical, morphological and physiological characteristics of the leaf. Similarly, the absorbed radiation can be used to drive photosynthesis, but it can also be dissipated as heat and fluorescence (Fig. 2). Even though only absorbed radiation has the potential to drive photosynthesis, leaf absorption cannot be optically detected by any direct approach. Both the amount of radiation absorbed and its spectral features have to be derived from the quantity and quality of the radiation that is being scattered from the upper or lower leaf surfaces (in terms of reflectance and transmittance), which can be optically detected and are influenced by the absorptive and scattering features of the leaf.

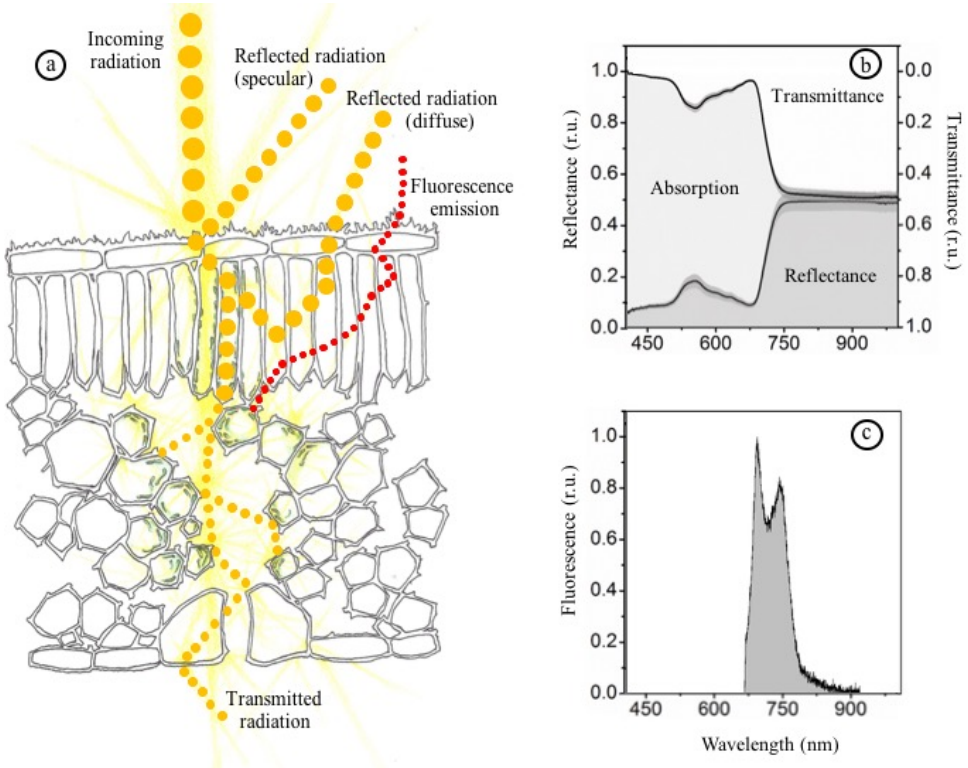

Figure 3. a) Illustration of the possible pathways of incoming radiation reaching a leaf. Part of the radiation is reflected at the surface (specular reflectance) and the remaining radiation enters the leaf. The scattering properties of the leaf make part of the radiation scatter back and exit the leaf as diffuse reflectance, whilst some is scattered forward and leaves the leaf as transmitted radiation. The incoming radiation that is neither reflected nor transmitted is absorbed, and a fraction of the absorbed radiation is emitted as fluorescence by the chlorophylls. **b)** Relative reflectance, transmittance and absorption spectra of a birch leaf, modified from Olascoaga et al. (2016). **c)** Chlorophyll fluorescence spectra from a birch leaf.

Biochemical constituents, such as leaf pigments, influence LOPs due to radiation absorption. For example, chlorophylls substantially influence LOPs in the visible region of the electromagnetic spectrum because of their abundance relative to the rest of the pigments. The peaks of the absorption spectrum of chlorophyll *a* are shifted slightly more towards shorter wavelengths of blue and longer wavelengths of red radiation than those of chlorophyll *b* (Blackburn 2007). Together both types of chlorophylls efficiently absorb over broad regions of the visible spectrum covering blue and red radiation, but are inefficient at absorbing green radiation. Additionally, carotenoids are pigments also capable of absorbing radiation to drive photosynthesis. The absorption peaks of many carotenoids cover the blue and green regions of the visible spectrum, but do not absorb in the yellow and red regions (Zur et al. 2000). Still, carotenoids extend the overall range of photons that can be absorbed and used to drive photosynthesis.

Non-photosynthetic pigments also absorb visible radiation that, despite not being used to drive photosynthesis, can also influence LOPs. Anthocyanins e.g. are non-photosynthetic pigments that absorb in the green-blue region of the visible spectrum (Merzlyak and Chivkunova 2000; Feild et al. 2001). Additionally, anthocyanins and other non-photosynthetic pigments, such as flavonoids, also absorb radiation in the ultraviolet (UV) region of the electromagnetic spectrum (Siipola et al. 2015), thus influencing LOPs beyond the visible region. Leaf constituents other than pigments also absorb electromagnetic radiation, e.g. H_2O, which efficiently absorbs radiation in the middle-infrared region of the electromagnetic spectrum (Jacquemoud and Baret 1990).

The refractive properties of the various leaf constituents influence LOPs by modifying the direction of propagation of the incident radiation. Morphological features at the leaf surface, as with epicuticular waxes and trichomes, reduce the quantity of radiation that can penetrate the leaf (and hence potentially be absorbed) due to their refractive properties. This is evident from the differences in LOPs generated by glaucous and non-glaucous leaves of the same species (Barker et al. 1997; Esteban et al. 2014) and the pubescent (i.e. leaf with trichomes) and glabrous (i.e. non-pubescent) leaves of the same species (Holmes and Keiller 2002). Morphological features defining the various tissues within a leaf can also influence LOPs. For example, the ability to propagate radiation within the leaf differs between palisade and spongy mesophylls (Vogelmann and Martin 1993; Johnson et al. 2005). Refractive discontinuities between the cell walls and intercellular air spaces also modify the direction in which radiation propagates. Refractive discontinuities along with the distribution of biochemical components and the various tissues along the cross-section of the leaf explain why LOPs also differ when assessed from the adaxial (i.e. upper surface of a leaf) and abaxial (i.e. lower surface of a leaf) sides of the same leaf (Vogelmann 1993; Lukeš et al. 2013). On a temporal scale, pigmentation and scattering properties of the leaf change along with the physiological status and ontogeny of the leaf (Seyfried and Schäfer 1983; Vogelmann 1993; Chavana-Bryant et al. 2016).

From an optical perspective, leaf reflectance represents the fraction of radiation incident on the leaf surface that is backscattered instead of being absorbed by the leaf components. Reflectance comprises a specular component dominated by the air-to-cuticle interface (Pfündel et al. 2006), which is polarised and has not penetrated the leaf, and a diffuse component that penetrates the leaf but is scattered back by structures and components within the leaf that the photon encounters. Similarly, transmittance represents the fraction of radiation incident on the leaf that is scattered forward, and it comprises the photons that are not intercepted by the various components and structures within the leaf. Thus, only the remaining fraction of photons incident on the surface of the leaf are not scattered backward (i.e. reflectance) or forward (i.e. transmittance), and these constitute the fraction absorbed by the various biochemical and morphological components of the leaf. As previously stated, only reflectance and transmittance can directly be detected by optical means. Thus, one of the most common methodologies for assessing leaf absorption is based on its indirect computation from reflectance and transmittance, which are often measured using an integrating sphere. This integrating sphere methodology is relatively straightforward with broadleaves. However, small-sized leaves with contrasting morphology compared to a broadleaf (e.g. needles) highlight the limits of this method, and make the accurate measurement of reflectance and transmittance (and hence the correct estimation of leaf absorption) challenging.

Reflectance and transmittance spectra of leaves include biochemical, morphological and physiological information that has the potential to detect species-specific features and

ontogenic and physiological differences. Optical features in leaf reflectance sometimes have the potential to discriminate between species. Daughtry and Walthall (1998) identified wavelengths along the green, red and near-infrared (NIR) regions of leaf reflectance spectra that could help to discriminate *Cannabis sativa* from trees (e.g. *Acer rubrum* and *Quercus rubra*) and herbaceous monocots (i.e. *Zea mays*). They suggested the possibility of optically discriminating well-fertilised and well-watered *C. sativa* plantations from surrounding vegetation. Similarly, Castro-Esau and colleagues (2006) were able to discriminate between tree species at different tropical sites in Mesoamerica based on selected wavebands of leaf reflectance and parametric and non-parametric classifiers. Nevertheless, they were not successful at detecting individuals of the same tree species at different sites based on the leaf-level spectral features of the given species, highlighting the influence of intra-specific leaf features (in terms of genetic, physiological and ontogenic factors) on LOPs.

Regarding intra-specific leaf features, the ontogenic state of the leaf can influence its leaf reflectance as a result of the dynamics within the pigments and internal structures (Merzlyak et al. 1999; Chavana-Bryant et al. 2016; Féret et al. 2017). A typical deciduous leaf turns from a light green colouration from budburst to a dark green colouration as the pigment concentration (particularly chlorophylls) and leaf thickness increase during development. As the leaf matures, reflectance in the visible region decreases along with an increase in pigment concentration, altering its reflectance especially in the green region. Leaf development also produces changes in NIR reflectance as the leaf thickens and its internal structures develop. In the senescing stage, green leaves turn into an orange-yellowish colouration, with an increase in reflectance along the visible region as chlorophylls degrade whilst carotenoids remain.

Apart from the developmental stage of the leaf, changes in the physiological status of a plant (e.g. due to stress conditions) can temporarily alter LOPs with respect to non-stressed conditions. Many biotic (e.g. fungal infection and mycorrhizal deficiency) and abiotic (e.g. herbicide and dehydration) stresses generally increase leaf reflectance in the green and red regions of the visible spectrum (Carter 1993) as a consequence of stress-induced chlorophyll degradation. The reduction in leaf water content caused by certain stressors (e.g. powdery mildew disease) further increases leaf reflectance in infrared regions affected by water absorption. High irradiance and drought stress can also influence LOPs in certain species by turning their leaves from a green to a red colour due to the synthesis of anthocyanins (Chalker-Scott 1999; Field et al. 2001) or rhodoxanthin (Gould 2004; Merzlyak et al. 2005).

A selection of wide and narrow wavebands along the visible and NIR regions of the electromagnetic spectrum reflected by vegetation allows the development of vegetation indices that can be used as optical proxies for plant stress and photosynthetic activity dynamics. These vegetation indices have traditionally been based on single reflectance wavebands, the differences between the reflectance of two wavebands, reflectance ratios, normalised ratios between the reflectance differences of two wavebands and derivatives from reflectance spectra (Le Maire et al. 2004; Gamon et al. 2004; Ustin et al. 2004). The normalised difference vegetation index (NDVI) is a widely used vegetation index. NDVI is based on changes in reflectance within the red region of the visible spectrum, which is sensitive to light absorption by chlorophylls. Therefore, one application is to use it as a proxy to determine the fraction of light absorbed by foliage (Fensholt et al. 2004), a parameter necessary for estimating gross and net primary production (Behrenfeld et al. 2001; Rahman et al. 2004; Running et al. 2004). Nevertheless, NDVI loses sensitivity for foliage with high chlorophyll concentrations and it is insensitive to changes in

photosynthetic light use efficiency (LUE). Alternative vegetation indices, such as the green normalised difference vegetation index (GNDVI), which is based on wavebands within the green region of the visible spectrum instead of the red region, have also been used (Gitelson et al. 1996).

The photochemical reflectance index (PRI) is another widely used vegetation index (Gamon et al. 1992; Soudani et al. 2014; Wong and Gamon 2015; Zhang et al. 2016). PRI is based on reflectance variations at a waveband of 531 nm, which have been correlated to thermal dissipation of the excitation energy via the VAZ xanthophyll cycle pigments (Gamon et al. 1992). The PRI also exploits an additional reference waveband at 570 nm, which is insensitive to VAZ pigment dynamics. PRI is thus a stress-related index that can track the thermal dissipation of the absorbed excitation energy and provide insight concerning LUE and photosynthetic activity dynamics. PRI can be used as an optical proxy for LUE, if the processes behind the thermal dissipation affecting the PRI signal are well understood. However, the total pool of pigments and the ratio between chlorophylls and carotenoids influence PRI at the seasonal scale (Filella et al. 2009), and hence also its relationship with LUE. Additionally, the wavebands selected to retrieve PRI at the leaf level might not be the appropriate wavebands when upscaling leaf reflectance to the shoots, canopies, etc. (Inoue et al. 2008; Garbulsky et al. 2011).

Reflectance from vegetation cannot be measured at the leaf scale only, but larger scales must also be involved (Mänd et al. 2010; Garbulsky et al. 2011; Zhang et al. 2016). This makes reflectance-based vegetation indices suitable candidates for monitoring the dynamics of canopy and ecosystem photosynthetic activity by optical means. Nevertheless, as upscaled reflectance spectra originate from each structure encountered by the optical detector, the detected radiation does not only emanate from the vegetation, but also from non-photosynthesising vegetation components (e.g. branches and tree trunks) and their surrounding environment (e.g. snow, rocks, ground, water streams, lakes, etc.) (Filella et al. 2004; Mänd et al. 2010). Radiation is also affected by canopy structure (Knyazikhin et al. 2013), and all of these factors must be accounted for to achieve accurate results (Hernández-Clemente et al. 2011; Zarco-Tejada et al. 2013). The chlorophyll *a* fluorescence signal, which is emitted from the photosystems of the chloroplasts within the leaf, is an alternative optical signal to the LOPs mentioned in this section, which is tightly linked to the photochemical apparatus and thus to the photosynthetic activity.

1.6.2 LOPs derived from emitted radiation: chlorophyll a fluorescence

Chlorophyll *a* fluorescence (hereinafter only fluorescence) is conceptually different to the above-mentioned leaf reflectance and transmittance of incident radiation, because fluorescence represents radiation emissions that have already been absorbed by chlorophylls. Thus it represents emitted radiation instead of reflected radiation. Due to energetic loss of the excited chlorophylls, the photons emitted as fluorescence are of longer wavelengths than those originally absorbed by the chlorophylls (Frankenberg et al. 2011; Porcar-Castell et al. 2014; Fig. 3). This loss of energy explains why the fluorescence spectrum, with emission peaks at approximately 690 nm and 740 nm emanating from PSII and PSI (Fig. 3), lies within the red and NIR regions of the electromagnetic spectrum (Meroni et al. 2009). Despite the *in vivo* fluorescence yield under steady-state illumination being only ~0.5–3% of the absorbed radiation (Krause and Weis 1991; Maxwell and Johnson 2000), fluorescence is a very powerful optical tool because its temporal dynamics

can be exploited for assessing photosystem-level energy partitioning (Maxwell and Johnson 2000). Hence we can gain insight on the dynamics of photosynthetic activity.

When a dark-adapted leaf is exposed to visible radiation, the photochemical pathway uses the absorbed excitation energy at sub-optimal rates because of differences in the activation rates of the light-dependent and light-independent photosynthetic reactions. In this scenario, the fluorescence signal emitted from the leaf initially registers a sudden increase. This initial increase is caused by the absorbed excitation energy being mainly diverted, so as to be emitted as fluorescence. The other two pathways to which the absorbed energy can be diverted (i.e. photochemical pathway and thermal dissipation pathway) are temporarily supressed immediately after exposure to irradiance. Nevertheless, if the fluorescence signal is monitored for a longer time after exposure to irradiance, the fluorescence signal is partially quenched (i.e. the fluorescence emission decreases) because an increasing fraction of the excitation energy is diverted to the photochemical pathway once it is activated. Additionally, every time the energy diverted to photochemistry exceeds the energy required to match the CO_2 assimilation rate, the imbalance between the light-dependent and light-independent reactions of photosynthesis triggers the activation of processes enhancing the thermal dissipation of the excitation energy. This also quenches the fluorescence signal, as it represents processes competing for the absorbed excitation energy. Fluorescence is therefore an LOP that allows monitoring the temporal dynamics of photosynthetic activity because the fluorescence signal can be quenched by both the photochemical pathway (detectable by the parameter PQ or photochemical quenching of fluorescence) and the processes that thermally dissipate the absorbed energy (detectable by the parameter NPQ or non-photochemical quenching of fluorescence).

As the energy absorbed by the photosystems can be diverted to three different pathways, a straightforward correlation between fluorescence and photochemistry cannot be established *a priori* because of the effect of thermal dissipation. However, a correlation between fluorescence and photochemistry can be achieved by a pulse- amplitude - modulated (PAM) fluorescence technique that utilises light-saturating pulses. These pulses are of such high light intensity that the photochemical pathway becomes momentarily saturated. However, this occurs so briefly that additional activation of processes that could thermally dissipate the excitation energy is not triggered. Thus, when a dark-adapted and non-stressed leaf receives a saturating light pulse, its fluorescence signal rises from a minimal (F_o) to a maximal (F_m). In this scenario, in which the leaf does not activate any process that could release the excitation energy as heat, F_o and F_m can be used to calculate the photochemical yield (ϕ_P) of the leaf, which will be maximal. However, when a saturating pulse is given to an illuminated or stressed leaf, the increase from a minimal to a maximal fluorescence signal is smaller, as different processes that thermally dissipate the excitation energy might be activated and could thus quench the fluorescence signal. PAM fluorometry in conjunction with saturating light pulses allows the deconvolution of the energy partitioning by NPQ and PQ parameterisation, making it possible to characterise the energy partitioning into photochemical and non-photochemical pathways by optical means. Beyond the characterisation of the energy partitioning, fluorescence appears to be a suitable candidate for the optical detection of gas-exchange -based photosynthetic parameters such as LUE. However, it is still necessary to test how well each of the fluorescence-based LOPs correlate with LUE at various spatial and temporal scales.

As fluorescence is an optical emission by the foliage, it can be detected at scales larger than the leaf. Nevertheless, most remotely sensed approaches for detecting fluorescence do not allow the use of light- saturation pulses (as previously explained for leaf-level

fluorescence detection), because the distance between the foliage and the detector is too large to successfully saturate the photochemical apparatus of the foliage. The most widely used remotely sensed fluorescence approach is therefore a passive approach that detects fluorescence emitted from the foliage due to solar radiation, i.e. so-called solar-induced fluorescence (SIF) (Meroni et al. 2009). SIF emissions must be extracted from foliage "apparent" reflectance spectra that are detected by means of platform-, airborne- and spaceborne-based reflectance detectors. SIF is retrieved from narrow regions of the reflectance spectra, in which solar irradiance has previously been absorbed either by the photosphere of the sun (i.e. Fraunhofer lines) or by the atmosphere of the Earth (i.e. telluric lines), making the radiation located at these narrow regions representative of foliage-emitted fluorescence. Hydrogen-α (650 nm) and potassium D1 (769 nm) bands are the most commonly exploited Fraunhofer lines, whilst O_2-B (690 nm) and O_2-A (760 nm) bands are the most commonly exploited telluric lines (Meroni et al. 2009; Porcar-Castell et al. 2014). The topical interest in using SIF to monitor photosynthetic activity at the global scale is shown by the recent selection of the FLEX Space Mission (Drusch et al. 2016) as the European Space Agency's 8th Earth Explorer Mission. However, unlike saturating pulse-based methods, SIF retrievals are dependent on the quantity of solar radiation and do not allow determination of the energy partitioning at the photosystem level. Directly correlating fluorescence to photosynthetic activity is also not an option. Hence work is in progress to relate passive and active fluorescence approaches (Magney et al. 2017), and is necessary to determine the physiological and non-physiological components affecting SIF at various spatial and temporal scales (Atherton et al. 2016).

In conclusion, reflectance-based and emission-based LOPs appear a promising tool for monitoring the temporal dynamics of photosynthetic activity, with the possibility of upscaling these approaches to targets larger than the leaf. Nevertheless, studying the spatial and temporal variations existing not only for the fluorescence signal but also for reflectance and transmittance spectra is still necessary, to ensure that changes in LOPs do in fact track changes in photosynthetic activity. Processes may still exist behind the dynamics of photosynthetic activity that the various LOPs do not fully capture, or that are captured using different sensitivities. Additionally, LOPs might not only miss physiological processes behind the dynamics of photosynthetic activity, but a component that might not be of physiological origin is also included within their signal.

2. AIM OF THE STUDY

The overall aim of this thesis was to investigate whether reflectance-based and fluorescence-based LOPs adequately represent biochemically, morphologically and physiologically-known processes that are behind the dynamics of photosynthetic activity, and whether the correlations between these optical and biochemical, morphological and physiological features hold true over various spatial and temporal scales.

This thesis hypothesised that our current theoretical framework to correlate LOPs and photosynthetic activity suffers from methodological and spatio-temporal limitations. The methodological limitations consist of as theoretical over-simplification of biochemical and physiological processes affecting photosynthetic activity and the LOPs. This thesis studied the over-simplification of the processes behind the absorption of radiation, which is mainly determined on a chlorophyll concentration basis. This was accomplished by assessing the

impact of needle epicuticular waxes on light absorption by describing the temporal dynamics of light reflectance by the epicuticular waxes of different-aged needles growing within the tree canopy (**Study I**). Additionally, instrumental/technical limitations were also defined as part of the methodological limitations that could affect a correlation between LOPs and the dynamics of photosynthetic activity. To reduce instrumental/technical limitations, an alternative method was developed for the accurate measurement of light absorption in leaves with complex geometry compared to leaf and needle light absorption estimates using various methodologies that indirectly compute light absorption from reflectance and transmittance spectra (**Study II**). As part of the spatio-temporal limitations affecting the correlation between LOPs and photosynthetic activity, temporal limitations were explored by studying the accuracy of estimating over time the relationship of LUE with the reflectance-based PRI parameter and with fluorescence-based parameters. This allowed better understanding of the relationship between PRI and fluorescence (**Study III**). Lastly, spatial limitations of correlating LOPs and photosynthetic activity were studied by characterising the spatial variability of reflectance- and fluorescence-based parameters in broadleaves and the needle-like leaves of trees growing in stands of various densities (**Study IV**).

3. MATERIAL AND METHODS

3.1 Study sites and plant material

The studies presented in this thesis were carried out under laboratory and field conditions. Most of the laboratory experiments were carried out at Viikki Campus, Helsinki, whilst the field experiments were carried out at the Station for Measuring Ecosystem-Atmosphere Relations (SMEAR-II) at Hyytiälä forestry field station (61°31’ N, 24°17’ E), approximately 200 km northwest of Helsinki. Hyytiälä forestry field station belongs to the boreal coniferous forest type, and Scots pine (*Pinus sylvestris* L.) is the most common tree species. The stand was established in 1962 by planting trees after treatments with prescribed burning and soil preparation (Hari and Kulmala 2005).

All species used in this thesis are tree species. Of these, Scots pine and Norway spruce (*Picea abies* (L.) H. Karst) are both evergreen coniferous species native to Europe. Scots pine was used in all the studies presented in this thesis, whilst Norway spruce was only used in Study IV. Silver birch (*Betula pendula* Roth.) is also a tree species native to Europe, but unlike Scots pine and Norway spruce, it is a deciduous broadleaf. Silver birch was used in three of the four studies presented in this thesis. These three species are representative of both subtypes of the southern boreal forest: the spruce-dominated subtype, and the pine and pine-birch -dominated subtype. Blue spruce (*Picea pungens* Engelm.) was additionally used as a control in two of the studies, and as a comparison against the above-mentioned species native to the Finnish boreal coniferous forest. Blue spruce is an evergreen coniferous tree species native to the Rocky Mountains of the United States of America, but individuals of this species grow at Viikki Campus.

3.2 Leaf reflectance measurements

Leaf reflectance measurements were conducted during all the studies, but reflectance was measured using two different methodologies.

3.2.1 Bidirectional reflectance factor, R_B

The common methodology used in all studies consisted of assessing leaf reflectance with a FieldSpec HH VIS-NIR (ASD Inc. Boulder, USA) sprectroradiometer attached to a contact probe (ASD Inc. Boulder, USA) through a fibre-optic bundle. This sprectroradiometer has a spectral range of 325–1075 nm, a sampling interval of 1.6 nm and 3.5 nm of full width at half maximum (FWHM). The contact probe housed a 6.5-W halogen light source and a fibre-optic bundle, both facing the window of the contact probe, oriented to the leaf sample. Despite the light source being positioned at 78° with respect to the leaf sample, the fibre optic sits at an angle of 55° with respect to the leaf sample surface. This allows the calculation of bidirectional reflectance distribution factors (R_B, Schaepman-Strub et al. 2006) of the leaf sample. In this methodology, the leaf sample was placed in a blackened dark-adaptation leaf clip (Hansatech instruments Ltd. Norfolk, UK). The leaf clip has a central 12.5-cm^2 Ø hollow where the window of the plant probe fitted perfectly. The leaf clip hollow has a central 1.5-cm^2 Ø hole that allows the sample to be exposed to the window of the plant probe.

Leaf sample reflectance was assessed by estimating the photon flux density detected when the leaf sample was present relative to the photon flux density when a Spectralon® white reference was present. This is defined by the equation:

$$R_{\mathrm{B}} = \frac{I_{\mathrm{S\_RB}}}{I_{\mathrm{W\_RB}}} \tag{1}$$

where R_B is the leaf sample's bidirectional reflectance factor, and I_{S_RB} and I_{W_RB} are the photon flux densities of the leaf sample and the white reference, respectively.

Leaf R_B in study IV was calculated by substituting the ASD contact probe with a FluoWat clip (Alonso et al. 2007), which also allows measuring fluorescence spectra from the same sample.

3.2.2 Hemispherical reflectance factor, R_H

Leaf sample reflectance in study II was assessed using the same spectroradiometer used for R_B attached to a 3-in. Ø integrating sphere (RTS-3ZC, ASD Inc. Boulder, USA). An integrating sphere has a spherical interior coated with a highly reflective material, e.g. barium sulphate. This allows the irradiance entering the interior of the sphere to be evenly distributed along its walls, thus making the photon flux density uniformly distributed along its inner surface. The surface of an integrating sphere has holes (i.e. ports), which can vary in size and number depending on the model, and to which the sample, irradiance source, detector, etc. can be attached. When a sample is placed on one of the ports of an integrating sphere, the geometry of the device allows measuring the sample's hemispherical reflectance factor (R_H) instead of its R_B. For this methodology, the leaf sample was attached to a black cardboard holder, which was placed in front of a port of the integrating sphere. In the case of needles, a composite leaf sample was built by arranging several needles side-by-side as a

flat mat, and the gap fraction between the needles was estimated by scanning the leaf sample and discriminating between pixels with leaf material and empty pixels. For characterisation of the leaf sample R_H, illustrated in Figure 3 of Study II, the photon flux density within the integrating sphere was registered under three different configurations. The port opposite to that holding a 10-W collimated halogen light source held the leaf sample in one of the three configurations. In another of the configurations, the port opposite to that of the light source port held a white reference panel. The port opposite to that of the light source port held a light trap in the last configuration. With the help of these three configurations, leaf sample reflectance was assessed as:

$$R_{\mathrm{H}} = \frac{\left(\frac{(I_{\mathrm{S\_RH}} - I_{\mathrm{STR\_RH}})\, R_{\mathrm{SP}}}{(I_{\mathrm{W\_RH}} - I_{\mathrm{STR\_RH}})}\right)}{1 - G_{\mathrm{F}}} \tag{2}$$

where R_H is the leaf sample's hemispherical reflectance distribution factor, and I_{S_RH}, I_{W_RH} and I_{STR_RH} are the photon flux densities of the sample, the white reference and the stray light reflectance configurations, respectively. R_{SP} is the reflectance of the Spectralon ® white reference, averaged for the PAR region, and G_F represents the gap fraction of the sample.

3.2.3 Reflectance-based vegetation indices PRI and GNDVI

In studies III and IV, leaf photochemical reflectance index (PRI) was calculated based on the reflectance values under certain wavelengths, as:

$$PRI_{\mathrm{n}} = \frac{R_{\mathrm{n}} - R_{570}}{R_{\mathrm{n}} + R_{570}} \tag{3}$$

R_{570} being the reflectance obtained at 570 nm wavelength, and R_n the reflectance obtained at 'n' nm wavelength: 'n' = 525, 531, 539 or 545 nm wavelength.

In study IV, GNDVI was calculated based on the reflectance values at certain wavelengths, as:

$$GNDVI = \frac{R_{780} - R_{550}}{R_{780} + R_{550}} \tag{4}$$

where R_{780} represents the reflectance obtained at 780 nm wavelength, and R_{550} the reflectance obtained at 550 nm wavelength. GNDVI values were then used as a proxy of leaf PAR absorption as in Gitelson et al. (1996).

3.3 Leaf transmittance measurements

Leaf transmittance measurements were included in Study II, and were obtained through the above-mentioned ASD spectroradiometer attached via a fibre-optic bundle to the above-mentioned ASD integrating sphere. The methodology was equivalent as that previously described for the hemispherical reflectance factor measurements, but leaf sample

hemispherical transmittance factor (T_H) measurements require three alternative configurations for the sample, white reference and stray light transmittance, as shown in Figure 3 of Study II. Leaf sample transmittance was obtained as:

$$T_H = \frac{\left(\frac{I_{S\_TH}}{I_{W\_TH} - I_{STR\_TH}}\right) - R_W G_F}{1 - G_F} \quad (5)$$

where T_H is the leaf sample's hemispherical transmittance factor. I_{S_TH}, I_{W_TH}, and I_{STR_TH} are the photon flux densities under the sample, white reference, and stray light transmittance configurations, respectively. R_W is the reflectance of the integrating sphere wall, and G_F is the gap fraction.

3.4 Leaf absorption measurements

Leaf absorption measurements were included in Study II, and were assessed using two methods: i) a method based on computing the absorption from reflectance and transmittance measurements and ii) a direct estimation of leaf absorption.

3.4.1 Indirect absorption

Indirect absorption measurements were computed following two methods. In the studies where leaf sample bidirectional reflectance factors (R_B) were assessed, leaf sample absorption (A_B) was computed from R_B after assuming zero transmittance, as $A_B = 1 - R_B$. This indirect method for assessing absorption has an inherent bias due to the zero transmittance assumption. This bias is particularly evident in leaves with thin cross-sections. Nevertheless, it is still a much faster method than any method using integrating spheres, and A_B measurements can still be used to track temporal dynamics in the absorption. Leaf sample absorption (A_H) was computed as $A_H = 1 - R_H - T_H$ in cases where both leaf sample R_H and T_H were assessed.

3.4.2 Direct absorption

Direct measurements of leaf absorption were conducted by attaching the ASD spectroradiometer via a fibre-optic bundle to a 4-in. Ø integrating sphere (LabSphere 4P-GPS-040-SF) with four orthogonally oriented ports. One of these ports was used to connect a secondary 2-in. Ø integrating sphere holding a halogen bulb to the main sphere, thus acting as the light source. The direct measurement of leaf absorption required measuring the photon flux densities within the integrating sphere under three different configurations, as illustrated in Figure 4 of Study II.

The spectroradiometric measurement of the photon flux density inside the sphere was initially recorded with the sphere empty apart for a white thread (I_W). A pre-set amount of leaves or needles were next sewn to the thread and the photon flux density (I_S) was recorded. Finally, the sample was sprayed with a black paint, repositioned within the sphere, and the photon flux density (I_B) within the sphere was once again recorded. The measurement of the photon flux densities under these three configurations allowed the direct calculation of absorption (A_T) as:

$$A_{\mathrm{T}} = \frac{(I_{\mathrm{W}} - I_{\mathrm{S}})\, I_{\mathrm{B}}\, A_{\mathrm{BLACK}}}{(I_{\mathrm{W}} - I_{\mathrm{B}})\, I_{\mathrm{S}}} \tag{6}$$

where A_{BLACK} was the absorption of the black paint on a piece of cardboard painted black, averaged for the PAR region and indirectly computed from T_{H} and R_{H}.

To define the amount of sample material to use, the saturation of the sphere was analysed by measuring the $I_{\mathrm{B}}/I_{\mathrm{W}}$ parameter under increasing areas of blackened sample material. The saturation of the sphere was defined as the part of the regression between $I_{\mathrm{B}}/I_{\mathrm{W}}$ and sample surface diverting from the linear regression. For each of the tested species, we used an amount of leaves or needles equivalent to the largest tested area with a value of $I_{\mathrm{B}}/I_{\mathrm{W}}$ that remained within the linear part of the saturation function.

3.5 PAM fluorescence measurements

A PAM fluorometer (Porcar-Castell et al. 2008) detects fluorescence emitted from a sample under natural light conditions, unaffected by the optical contamination produced by the ambient light. By applying saturating light pulses, PSII-level energy partitioning can be calculated by means of the minimal (F) and maximal (F'_{m}) fluorescence signals associated with each of the pulses (in the dark-adapted cases F becomes F_{o}, and F'_{m} becomes F_{m}). Based on these fluorescence values, the associated PSII photochemical yield (Φ_{P}) can be calculated as:

$$\phi_{\mathrm{P}} = 1 - \left(\frac{F}{F'_{\mathrm{m}}}\right) \tag{7}$$

Using these fluorescence values, the NPQ of the fluorescence signal, a parameter associated with the thermal dissipation of the absorbed excitation energy, was calculated as:

$$NPQ = \left(\frac{F_{\mathrm{mR}}}{F'_{\mathrm{m}}}\right) - 1 \tag{8}$$

where F_{mR} is the reference F_{m}, representing the F_{m} value associated with the maximum yield of photochemistry ϕ_{Pmax} (Eq. 7). For each NPQ value, the associated PQ of the fluorescence signal was also calculated as:

$$PQ = \left(\frac{F_{\mathrm{mR}}}{F}\right) - \left(\frac{F_{\mathrm{mR}}}{F'_{\mathrm{m}}}\right) \tag{9}$$

Finally, the photochemical electron transport rate (ETR) along the photosystems was calculate as:

$$ETR = \mathrm{PAR\ A\ } \phi_{\mathrm{P}}\ 0.5 \tag{10}$$

where PAR represents the photon flux density (µmol photons m^{-2} s^{-1}) within the photosynthetically active radiation region, A represents leaf absorption, ϕ_{P} is the

photochemical yield and 0.5 represents the fraction of electrons associated with each of the two photosystems.

PAM fluorescence measurements were conducted in Study III using an FMS-2 portable fluorometer (Hansatech instruments Ltd. Norfolk, UK), whilst the characterisation of PSII energy partitioning via fluorescence in Study IV was conducted with the PAM fluorometer equipped within a GFS-3000 IRGA device (Heinz Walz GmbH, Effeltrich, Germany) that analyses gas exchange between leaves and the atmosphere. Both PAM fluorometers integrate the fluorescence signals for its entire spectrum, thus giving a single value of fluorescence instead of its spectral profile.

3.6 Fluorescence spectral measurements

Fluorescence spectral measurements were performed in Study IV, and obtained with a FluoWat clip (Alonso et al. 2007) attached via a fibre-optic bundle to an ASD handheld spectrometer (PANlytical Inc., Boulder, USA). The FluoWat clip is a probe that houses a sample holder and a fibre optic bundle, but it also has an additional compartment for housing a filter. In this study, we used a low-pass 650-nm filter (650 nm OD 4, short pass filter, Edmund Optics Ltd., York, UK). The illumination source of the FluoWat clip consisted on an external halogen bulb (equivalent to the one used for the 2-in. Ø LabSphere integrating sphere) oriented towards the FluoWat clip, and following the same geometry as that of the ASD contract probe. The FluoWat clip was also used to obtain leaf sample R_B, as previously explained, but when the 650-nm filter was placed in front of the illumination source, it detected the directional fluorescence spectrum beyond 650 nm. To measure R_B and fluorescence spectra from the needles of evergreen species, needle mats with a thickness of one needle were constructed by placing the needles side-by-side with small gaps in-between. The gap fraction of the sample, estimated as the proportion of needle samples to gaps between needles, was estimated using scanning images of the needle mats and a custom Python script. Fluorescence spectra were normalised to the sum of the radiation emanating from a white reference, and smoothed using a 1D Gaussian filter.

3.7 Gas exchange measurements

3.7.1 GFS-3000 infrared gas analyser

Gas exchange measurements in Study IV were obtained using a GFS-3000 infrared gas analyser. Some foliage was placed in the measuring chamber under controlled temperature and humidity conditions, and exposed to various PAR intensities ranging from darkness to a photon irradiance of 1200 µmol photons m^{-2} s^{-1}. This was performed by means of a LED-array/PAM-fluorometer 3055-FL light source (Heinz Walz GmbH, Effeltrich, Germany) consisting of 24 red (peak at 640 nm wavelength) and two blue (peak at 470 nm wavelength) LEDs. Results were then used to model CO_2 assimilation as a function of light intensity by means of a rectangular-hyperbolic function (Kolari et al. 2014).

3.7.2 Automatic shoot chambers

The shoot chambers used in Study III consisted of cylindrical chambers of plastic in which the foliage was placed (Kolari et al. 2009). The chambers were automatic and were kept open except when gas exchange measurements were recorded. During recording the chambers were sealed for a minute, and foliage transpiration and CO_2 assimilation rates along with light intensity and temperature were registered. Shoot chamber measurements were used to compute photosynthesis LUE. LUE was defined as the sum of the assimilation and respiration rates, divided by light intensity. For each of the dates under examination, the respiration rate was derived from the night-time respiration rates (including one week before and after the measurement day) by applying a linear regression model against temperature, and assuming that daytime respiration responses to temperature are maintained at approximately the same rate. LUE was averaged over a five-day window around each of the sampling dates to compare the shoot chamber measurements with the additional reflectance- and fluorescence-based optical measurements conducted for this study. Only pre-noon and gas-exchange data obtained for photon irradiances lower than 400 μmol photons m^{-2} s^{-1} were taken into consideration.

4. RESULTS AND DISCUSSION

As previously stated, biochemical and physiological processes affecting the light-dependent reactions of photosynthesis influence LOPs, which can be optically detected. Therefore a theoretical framework supports the correlation of LOPs with the light-dependent reactions of photosynthesis. Furthermore, as the light-dependent and light-independent reactions of photosynthesis tend to reach a photostatic balance, LOPs can be used as optical tools for monitoring the dynamics of photosynthetic activity. However, methodological and spatio-temporal limitations may affect the correlation between LOPs and photosynthetic activity. The amount of radiation absorbed by leaves and the partitioning of the absorbed energy into photochemical and non-photochemical processes explain the dynamics of photosynthetic activity. At the seasonal scale, the photosynthetic activity of a deciduous species is largely driven by the amount of radiation absorbed by its leaves. Reflectance-based vegetation indices, such as NDVI (Sjöström et al. 2009; Ulsig et al. 2017) and GNDVI (Gitelson et al. 1996), can be used as optical proxies to estimate the amount of radiation absorbed by foliage. Nevertheless, the amount of foliage does not temporarily change as drastically in evergreen species as in deciduous species. Thus, the photosynthetic activity of evergreen leaves is largely driven by the efficiency at which light is allocated into photosynthesis instead (Garbulsky et al. 2008).

Biochemical and physiological processes affecting the amount of energy available for photosynthesis correlate with reflectance-based optical parameters. The epoxidation and de-epoxidation of the VAZ xanthophyll cycle, which has been linked to the thermal dissipation of the absorbed energy, correlate with reflectance-based PRI (Gamon et al. 1992). PRI can also be used to estimate photosynthetic LUE (Peñuelas et al. 1995). Certain biochemical and physiological processes affecting the amount of radiation available for photosynthesis correlate with chlorophyll fluorescence-based optical parameters. This is the case with the partitioning of the absorbed energy into photochemical and non-photochemical pathways, which can be assessed with the PQ and NPQ parameters (Maxwell and Johnson 2000;

Porcar-Castell 2011). The fraction of the absorbed energy diverted into photochemistry can also be assessed by means of the fluorescence-based ϕ_P (Maxwell and Johnson 2000). Fluorescence-based parameters (e.g. Fv/Fm, ϕ_P) can also be used to estimate photosynthetic LUE (Peñuelas et al. 1995). Additionally, the combination of both reflectance- (e.g. absorption of radiation) and fluorescence-based optical parameters (e.g. ϕ_P) can also be used to determine parameters related to photosynthetic activity, e.g. ETR (Maxwell and Johnson 2000). Even certain reflectance-based optical parameters (e.g. PRI) can correlate with additional optical parameters that are based on fluorescence (e.g. NPQ and ϕ_P) (Peñuelas et al. 1995; Ač et al. 2009; Garbulsky et al. 2011).

Our understanding of the dynamics on photosynthetic activity and LOPs thus supports a theoretical framework for assessing photosynthesis by optical means. Nevertheless, two types of limitations, i.e. methodological and spatio-temporal limitations, can still affect this framework. Certain methodological limitations comprise the over-simplification of biochemical and physiological processes that correlate photosynthetic activity dynamics with LOP dynamics. For example, this thesis shows that leaf reflectance varies due to the reflectance properties of epicuticular waxes, which can consequently affect the amount of radiation absorbed by leaves, a parameter that several previous studies have considered constant or mainly pigment concentration -dependent (Carter and Spiering 2002). Other methodological limitations are instrumental and technical by nature. These were addressed in this thesis by comparing alternatives for computing the absorbed radiation for leaves of contrasting morphology, which is particularly challenging and error-prone for small-sized leaves and for morphologies that differ from typical broadleaves (Daughtry et al. 1989; Middleton et al. 1996; Mesarch et al. 1999).

Spatio-temporal limitations make up the other type of limitation in our theoretical framework. This thesis addressed temporal limitations by studying the correlations between NPQ and PRI, and NPQ and fluorescence-based ϕ_P at various temporal scales. This thesis shows that fluorescence- and reflectance-based LOPs present a "baseline" component, which depends on both the light environment where the leaf developed and the species. Thus, this "baseline" component is disengaged from LOP dynamics associated with biochemical and physiological processes affecting photosynthetic activity.

Overall this thesis deepens our knowledge of leaf optical properties within the visible region of the electromagnetic spectrum and increases our existing knowledge concerning the relations between LOPs and the physiological and non-physiological processes influencing photosynthetic activity.

4.1 PAR absorption dynamics in leaves

Optical leaf properties are affected by pigments, structural leaf components and leaf water content. Pigments within the leaf mainly modify reflectance, transmittance and absorption spectra along the visible region of the electromagnetic spectrum, whilst structural components, such as cell walls and the leaf water content, also modify reflectance, transmittance and absorption spectra along the NIR and short-wave infrared regions, respectively (Govender et al. 2009).

Although LOPs within the PAR region are almost completely described by the concentration and dynamics of chlorophyll and accessory pigments, this thesis shows that additional constituents apart for pigments (e.g. epicuticular waxes) also play a role in LOPs, with variable significance under various spatio-temporal scales. They contribute to

modulating the fraction of absorbed radiation available for photosynthesis. LOPs are thus a complex product of both pigment dynamics and dynamics of non-pigment leaf constituents.

Study I addressed the effect that epicuticular waxes had on the optical properties of Scots pine needles. Needle PAR reflectance was measured as a proxy of PAR absorption to overcome the difficulties in directly measuring PAR absorption in needle-like leaves (a methodological limitation that it was addressed in Study II). Results show that the foliage of boreal conifers, such as Scots pine, modulate the fraction of PAR that they reflect due to seasonal dynamics of the optical properties of the epicuticular waxes. In fact, light reflectance by epicuticular waxes is reduced to a minimum as wintertime approaches, and increases from spring to summer. This study did not assess the reasons behind the optical dynamics of epicuticular waxes. The optical dynamics of Scots pine epicuticular waxes could be due e.g. to structural changes in the crystallites of epicuticular waxes and the regeneration of epicuticular waxes (Sase et al. 1998; Neinhuis et al. 2001), but further studies are necessary for discovering the factors affecting the optical dynamics of Scots pine epicuticular waxes shown in Study I.

From a physiological viewpoint, differences in gas-exchange and photoinhibition dynamics between glaucous and non-glaucous individuals of a same species already suggest that leaf epicuticular waxes may play a photoreceptive role in addition to other functions (Mohammadian et al. 2007). The seasonal variations observed in PAR reflectance by Scots pine epicuticular waxes were modest in this study. Nevertheless, the existence of a seasonal dynamics extends the rationale of the photoprotective role of epicuticular waxes to the temporal scale. For the case of boreal Scots pine, increasing the fraction of radiation reflected from the needles from winter to spring could reduce the excitation pressure on their photochemical apparatus during the bright but cold days of spring, when radiation is absorbed in excess for the thermally-suppressed enzymatic reactions of photosynthesis (Ensminger et al. 2004; Porcar-Castell 2011). Nevertheless, this hypothetical photoprotective role of epicuticular waxes at the seasonal scale still needs to be explicitly tested.

The seasonal dynamics of PAR reflectance by epicuticular waxes were masked by the superimposed effects that needle ageing and wax weathering have on foliage reflectance. This is why seasonal dynamics of PAR reflectance by epicuticular waxes were only detectable in the youngest cohorts of needles out of the three cohorts in the Scots pines from southern latitudes of the boreal forest. The magnitude of the seasonal dynamics on epicuticular wax reflectance is small relative to the variability introduced by environmental factors, but these seasonal dynamics may be more apparent in individuals with larger epicuticular wax loads and in individuals with a larger number of needle cohorts, e.g. trees growing in more northern and subarctic latitudes (Bäck et al. 1994; Dengel et al. 2013). Whether seasonal dynamics of PAR reflectance by epicuticular waxes are enhanced or diminished in individuals growing at different latitudes, such as those in the Mediterranean region, remains to be seen. In this region both the wintertime along with summertime temperature and water stressors may affect not only photosynthetic performance, but also the optical dynamics of epicuticular waxes. The contribution of epicuticular waxes to PAR reflectance of Scots pine needles was just 3–4% in Study I, but it contributed more than 10% for blue spruce. These differences suggest that optical property dynamics by epicuticular waxes may also vary for the different species, and thus it is important to consider them as a physiological process behind LOP dynamics.

As photosynthesis requires radiation for the transformation of CO_2 into sugars, measuring the fraction of PAR absorbed by a leaf is a parameter of paramount importance

in many studies dealing with photosynthesis. This parameter is included in equations related to photosynthesis such as photosynthetic ETR or LUE. However, the processing calculations for data obtained from commercial fluorometers and infrared gas analysers are automatically set to use 0.84 as the value of PAR absorption (Baker 2008), e.g. when calculating photosynthetic ETR and LUE. This thesis shows that PAR absorption not only differs between species (Study II), but can also temporally and spatially differ within the foliage of a single species (Studies I and IV).

When making generalisations concerning photosynthesis, it is not practical to measure leaf-level PAR absorption for all the leaves in a canopy. Therefore, assuming all the leaves studied are relatively similar (and hence their PAR absorption is also similar), parameters, such as photosynthetic ETR (Eq. 10), can be estimated by simply multiplying a general value for photochemical yield by the light intensity (Maxwell and Johnson 2000). However, this study suggests that efforts to estimate individual leaf light absorption are worthwhile, especially in studies comparing parameters related to the photochemical activity of various species (Pieruschka et al. 2010; Magney et al. 2017), or between the leaves of a particular species that differ in age, canopy position or season. This is true because PAR absorption can greatly differ from the commonly assumed 0.84, and thus obscure the actual dynamics of photosynthesis. As an illustrative example, Figure 4 shows differences in the estimation of photosynthetic ETR on the needles of blue spruce, when needle PAR absorption is set to A=0.84 (blue line) and when its actual needle PAR absorption value (i.e. A=0.676) is computed by means of an integrating sphere.

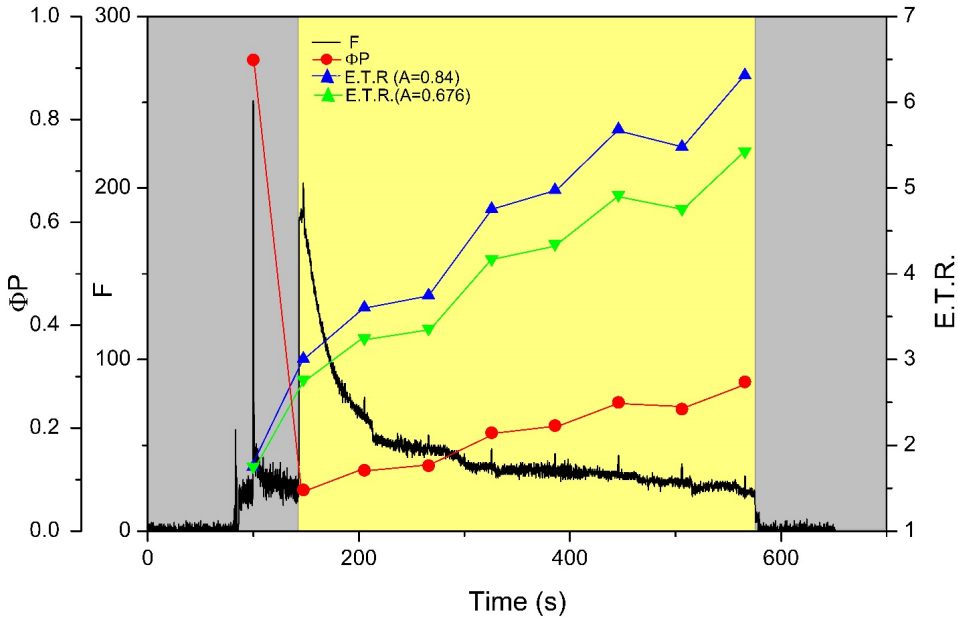

Figure 4. Temporal dynamics of the photosynthetic electron transport rate (ETR, Eq. 10), derived from chlorophyll *a* fluorescence data, for young blue spruce (*Picea pungens*) needles, either assuming a PAR absorption value of 0.84 (A=0.84, blue line), or calculating

PAR absorption through an integrating sphere (value taken from Study II, A=0.676, green line). The grey areas correspond to dark conditions, and the yellow areas correspond to illuminated conditions with a constant PAR intensity of 50 μmol m^{-2} s^{-1}. *F* = Chlorophyll *a* fluorescence, ϕ_P = Photochemical yield.

4.2 Methods for assessing PAR absorption in leaves

The most common method for estimating radiation absorption by a leaf is by indirectly computing it from leaf measurements of radiation transmittance and reflectance, which, unlike absorption, can be detected by optical means. However, leaf reflectance represents a complex optical signal with a specular and a diffuse component, a consequence of the scattering properties of structures both at the surface (specular component) and interior of the leaf (diffuse component) (Pfündel et al. 2006).

When a structure reflects a large diffuse component, its scattering properties remain relatively unchanged irrespective of the angle of detection. Thus the reflectance of radiation detected in a single direction is legitimately representative of its three-dimensional scattering properties. Leaf PAR absorption can be inferred from reflectance-based vegetation indices (Xiao et al. 2004**)** such as the GNDVI used in Study IV. Nevertheless, the complexity of leaf scattering properties make more-demanding hemispherical, multidirectional detection via an integrating sphere a more-appropriate candidate for assessing the three-dimensional scattering properties in leaves along with its associated absorption.

The most common method for computing leaf absorption via an integrating sphere is the 'external' method, in which the sample is placed on the measuring ports located over the surface of the sphere, allowing the calculation of leaf reflectance and transmittance. This 'external' method is relatively easy for broadleaves, but challenging to use with needles, due to their size and three-dimensional shape. Thus, to fully cover the measuring port of the integrating sphere, a composite needle sample is often built by placing needles side-by-side to form a mat. The needles have unavoidable gaps between them. Consequently, the error introduced in leaf reflectance and transmittance spectra due to the gap fraction of the leaf sample needs to be corrected (Eqs. 2 and 5), which is not always a straightforward task (Daughtry et al. 1989; Middleton et al. 1996; Mesarch et al. 1999).

Study II presents an alternative 'internal' method for measuring leaf and needle absorption, which is independent of the reflectance and transmittance spectra measurements. The method, which consists on painting the sample black and measuring the outgoing photon flux density from the integrating sphere before and after painting, was very useful for needles, as it by-passes the need to calculate the gap fraction that otherwise affects the accuracy of transmittance and reflectance spectra (Daughtry et al. 1989; Middleton et al. 1996; Mesarch et al. 1999). Thus, the methodology presented in Study II is expected to facilitate the calculation of leaf PAR absorption, and help avoid the use of constant PAR absorption values in studies dealing with the dynamics of photosynthetic activity (Pieruschka et al. 2010; Magney et al. 2017).

Although the 'internal' method presented in this study does not allow the characterisation of reflectance and transmittance spectra, it can still be used as a benchmark for studies requiring the reflectance and transmittance spectra of needles, because this method provides an independent measurement of leaf absorption that can be compared against values computed from the reflectance and transmittance spectra using the 'external'

method. Thus, the 'internal' method not only improves the calculation of leaf and needle absorption, but also the accuracy of reflectance and transmittance spectra characterisation.

4.3 LOP sensitivity to physiological processes behind the dynamics of photosynthesis

Apart for instrumental and methodological limitations, our theoretical framework for correlating LOPs with the physiological processes behind the dynamics of photosynthetic activity may not hold true when extended to various temporal scales. Temporal limitations to our theoretical framework can indicate an over-simplified approach to characterising biochemical and physiological processes behind the dynamics of photosynthesis. However, they can also demonstrate that certain optical parameters derived from LOPs have different sensitivities to biochemical and physiological processes influencing photosynthetic dynamics. Study III investigated whether reflectance- and fluorescence-based optical parameters could successfully track the seasonal dynamics of photosynthesis.

At the diurnal scale, when a leaf absorbs radiation in excess, the main fraction of the excess energy is thermally released by the de-epoxidation of the VAZ xanthophyll pigments. The de-epoxidation state of the VAZ pigments is reversible and the thermal dissipation is deactivated when the absorbed radiation is not in excess anymore. Thus, the epoxidation state of the VAZ cycle affects photosynthetic LUE, as it modifies the fraction of absorbed energy available for photosynthesis. At the seasonal scale, the thermal release of the absorbed excitation energy shows a sustained component during unfavourable seasons (e.g. winter) when VAZ pigments are kept in a de-epoxidised state that does not revert in the short term. The epoxidation state of the VAZ pigments produces changes in leaf reflectance at a wavelength of 531 nm, which has been used to develop the vegetation index PRI (Gamon et al. 1992) as an optical proxy for photosynthetic LUE changes (Peñuelas et al. 1995; Filella et al. 1996; Garbulsky et al. 2011). However, PRI is also affected in the long term by the total pool of carotenoids and the ratio between carotenoids to chlorophylls present on the leaf (Filella et al. 2009). As LUE is a parameter linking the amount of carbon assimilated by a leaf to the amount of radiation absorbed by the leaf, estimating LUE along with the fraction of absorbed PAR allows the calculation of photosynthetic activity in terms of gross primary production (GPP), upscaling the dynamics of photosynthesis to scales broader than the leaf by means of PRI.

Reflectance-based PRI was used in Study III as a proxy of LUE and their relationship was investigated at the seasonal scale. An alternative widely used methodology for the characterisation of energy partitioning (which can provide insight on LUE) is based on the study of the PQ (Eq. 9) and NPQ (Eq. 8) parameters from the fluorescence signal that emanates from the photosystems within the foliage. NPQ is a fluorescence-based parameter that is a proxy of the heat dissipation of the absorbed excitation energy, especially via epoxidation and de-epoxidation dynamics of the VAZ cycle pigments. This theoretical framework supports a correlation between PRI and NPQ. But how well do both optical approaches track the processes controlling energy partitioning at the seasonal scale?

For boreal evergreen foliage, our results show that the seasonal correlation between reflectance-based PRI and fluorescence-based NPQ breaks during the early spring, when PRI is not sensitive enough to detect an increase in thermal dissipation of the absorbed excitation energy. Thus, PRI underestimates NPQ and so overestimates the photochemical yield of evergreen foliage during early spring, attributing a larger photosynthetic capacity than when estimated by means of fluorescence. The disassociation between these two

optical parameters highlights the fact that despite PRI and NPQ correlating with the thermal dissipation of the absorbed energy, physiological processes controlling each of the parameters differ, and the temporal contributions of these processes also differ when characterising the photochemical state either by reflectance- or fluorescence-based parameters. In fact, the fluorescence-based NPQ parameter correlated more strongly with the de-epoxidation state of the VAZ cycle carotenoids than with the carotenoid pool size, whilst reflectance-based PRI correlated more strongly with the carotenoid pool size than with the de-epoxidation state of the VAZ cycle carotenoids. This result also reveals that, during early spring, a fraction of NPQ is still not explained by the de-epoxidation state of the VAZ cycle carotenoids, showing that our understanding of the physiological processes behind the temporal dynamics of NPQ remains incomplete. We suggest that the winter aggregation of the light-harvesting complexes could partly explain the NPQ that is independent of the VAZ cycle dynamics, as light-harvesting complex aggregation enhances the thermal dissipation capacity of overwintering Scots pine foliage (Ottander et al. 1995; Ensminger et al. 2004).

From an optical viewpoint, the correlation between PRI and NPQ during the whole-year period increased when applying spectral forms alternative to the standard form of PRI (i.e. 531 nm wavelength reflectance). When substituting reflectance from a wavelength of 531 nm by reflectance from wavelengths 539 nm and 545 nm, the correlation between PRI and NPQ increased from 0.83 to 0.86 in both alternative cases. Despite the three PRI spectral forms showing a similar correlation with NPQ during the growing season period, for early spring the two alternative spectral forms also correlate more strongly with NPQ than the standard form of PRI. This suggests that these alternative PRI forms could actually be more sensitive at detecting physiological processes behind the xanthophyll-independent sustained form of NPQ. These discrepancies between PRI forms highlight the complexity behind LOPs, and allow new alternatives for the characterisation of heat dissipation from reflectance-based PRI to be suggested. From a practical perspective, alternative PRI forms compared to the ones investigated in Study III are better suited for tracking the seasonal dynamics of NPQ in boreal evergreen foliage.

When upscaling from the photochemical to the photosynthetic scale, which involves more physiological processes, the successful optical detection of many of the processes behind photosynthetic dynamics still remains a challenge. Apart for the correlation between the fluorescence-based maximum yield of photochemistry (ϕ_{Pmax}) and LUE, ϕ_{Pmax} values of 0.83, which are representative of leaves with non-stressed photosystems (Johnson et al. 1993), correlated poorly with very variable LUE values. This range of LUE values associated with almost a single ϕ_{Pmax} value, most likely represents the effect of physiological processes decoupling the energy balance between light-dependent and light-independent reactions of photosynthesis. Processes, such as photorespiration (Niinemets et al. 1999; Wingler et al. 2000), can cause leaves with similar photochemical quantum yields to have contrasting CO_2 assimilation rates due to the modification of the carboxylation and oxygenation ratio of the rubisco enzyme.

In Study III, PRI and LUE correlated when considering the whole-year period, but showed poor correlation when the data were analysed only for the growing season period or the cold period. From a physiological viewpoint, the variation in growing season LUE could be explained by the contribution of physiological processes that represent alternative energy sinks to that of CO_2 assimilation. These alternative energy sinks were not investigated in this study, but future work could be done to investigate their role when correlating LOPs to photosynthetic dynamics. Over-simplifying the role of certain known

physiological processes (e.g. the assumption of constant leaf PAR absorption over the entire growing season) that are expected to vary in the temporal scale used in this study, can also add variability to the relationship between PRI and LUE.

This study also highlights that studying the dynamics of photosynthesis by optical means should combine reflectance- and fluorescence-based optical parameters for a more complete insight into energy partitioning, as reflectance- and fluorescence-based optical parameters show different sensitivities to the biochemical and physiological processes behind photosynthetic dynamics. More complete studies should be performed for the seasons or temporal conditions in which the correlation between both types of optical parameters break, paying special attention to the characterisation of physiological processes that may not have been considered relevant.

4.4 Physiological and non-physiological LOP components

The use of reflectance- and fluorescence-based parameters as optical proxies for biochemical and physiological processes behind photosynthetic dynamics has already been demonstrated in Studies I and III of this thesis. However, in Study IV we investigated whether LOPs could also include a non-physiological "baseline" component detachable from the physiologically driven facultative and constitutive components of the signal. We hypothesised that this "baseline" component would most likely be associated with morphological characteristics of the leaf, which could depend on species and on the spatially dependent light environment in which the leaf develops. Thus, we investigated the spatial variation of LOPs in various tree species growing at various stand densities.

Study IV revealed that PRI is strongly dependent on species. PRI also depends to a lesser extent on the light environmental conditions in which the leaves develop, which is influenced by leaf canopy position and stand density. This study shows that correlations between LOPs and the dynamics of photosynthesis are thus also subjected to species and spatial constraints. This species dependency of LOPs is also in agreement with results in Study II concerning leaf and needle reflectance spectra of various species. The spatial dependency of LOPs is also partly implicit from the epicuticular wax reflectance of needles presented in Study I, which were acclimated to different light environments within the canopy and varied on the fraction of reflected radiation by their epicuticular waxes. Interestingly, the "baseline" component associated with PRI segregates broadleaved and needle-leaved species. The existence of segregation between broadleaves and needles by PRI will definitely have an effect on the characterisation of photosynthesis via reflectance-based optical parameters when the study scale exceeds the leaf level in stands comprising both broadleaved and needle-leaved species. Thus, in addition to accounting for the non-photosynthetic background component introduced to the reflectance signal detected for scales larger than the leaf (Mänd et al. 2010), efforts should also be made to disentangle the "baseline" component of the optical signal that it is related to the species- and spatial-specific characteristics of the foliage.

The reflectance-based leaf GNDVI values (Gitelson et al. 1996) reported in this study correlated with leaf PAR absorption values obtained via integrating sphere measurements following the methodologies presented in Study II. However, GNDVI did not correlate with leaf and needle pigment contents. As stated in our study, such mismatches have a direct repercussion on the estimation of leaf biochemical components through optical means, because they rely on pre-established relationships such as that between chlorophyll

concentration and the fraction of PAR absorbed by the leaf. This result highlights once again the limitations of over-simplifying the physiological processes behind LOPs.

Alternatively, "baseline" components of the optical parameters derived from the fluorescence signal, which emanate from the photosystems within the chloroplasts and thus present a narrower footprint than that of reflectance-based optical signals, were less variable between species than the reflectance-based signal. However, the ratio F_{690}/F_{740}, which is related to leaf PAR absorption (Gitelson et al. 1998; Buschmann 2007), remained species-dependent. The results of Study IV in conjunction with Study III suggest that fluorescence-based optical parameters might be better suited for assessing correlations with photosynthesis than reflectance-based optical parameters, not only at the leaf level, but presumably also at the canopy, stand and even larger levels. However, the PAM methodology used in this thesis to capture dynamics of photosynthesis is not applicable at scales larger than the leaf, whereas reflectance-based approaches are.

PAM methodology requires the sample to be placed at a close distance to the source of excitation, and thus it is an impracticable methodology for distances longer than a few centimetres. An alternative active fluorescence methodology based on fast repetition rate fluorometry (Kolber et al. 1998) and known as laser-induced fluorescence transient (LIFT) can monitor the dynamics of photosynthesis at a distance of 5–50 m from the target, spatially upscaling the estimation of photosynthesis by means of fluorescence up to the canopy level (Pieruschka et al. 2010; Raesch et al. 2014). LIFT has been shown to successfully track the temporal dynamics of photosynthesis in various species responding to various stressors, but the absolute values of its optical parameters differ from those obtained through PAM methodology (Pieruschka et al. 2010; Raesch et al. 2014). This difference is to a large extent explained by the fact that whilst PAM methodology uses saturating pulses that reduce the entire population of PSII RCs, LIFT methodology yields only a partial reduction in the entire population of PSII RCs, meaning that the estimation of maximal fluorescence signal should be calculated by means of a model approach (Kolber et al. 1998). Alternatively, canopy architecture also appears to affect LIFT measurements, and the detector's optical footprint (e.g. 10 cm Ø) may contain many layers of the canopy, adding noise to the signal (Raesch et al. 2014).

An additional fluorescence methodology can be used for upscaling to scales larger than the canopy. Unlike PAM and LIFT, this approach is not based on an active excitation source, but relies on the fluorescence emissions induced by solar radiation, and so it is known as solar-induced chlorophyll fluorescence (SIF) (Meroni et al. 2009). The SIF emission is added to the superimposed radiation reflected from the sample, thus contributing to the optical signal that is instrumentally detected (Zarco-Tejada et al. 2000). As SIF is included in this "apparent" reflectance signal (Meroni et al. 2009), fluorescence emissions can then be measured from the leaf scale up to the satellite scale. Nevertheless, it is still necessary to separate the fluorescence signal from the "apparent" reflectance signal. The different approaches for estimating SIF are reviewed in Meroni et al. (2009). Future missions, such as the FLEX Satellite Mission (Drusch et al. 2016), estimated for launch in 2022, will seek to improve the spatial and spectral resolution of SIF detection. In recent years, global observations of GPP have positively correlated with the SIF signal, but their correlation weakens during the summer period for boreal vegetation, as needle-leaved evergreen forests have ~30% lower fluorescence than predicted (Frankenberg et al. 2011). On the other hand, it is still necessary to understand the physiological and non-physiological components affecting the dynamics of SIF over time and space.

Continuing to research the physiological and non-physiological components affecting the process of photosynthesis through each of these two optical approaches is crucial, along with studying the way in which reflectance- and fluorescence-based parameters correlate with each other and with the dynamics of photosynthesis.

5. CONCLUDING REMARKS

Reflectance- and fluorescence-based LOPs correlate with biochemical and physiological processes behind the dynamics of photosynthesis. However, the theoretical framework has methodological and spatio-temporal constraints, which weaken the correlations between LOPs and photosynthetic dynamics.

This thesis deepened the knowledge on methodological constraints as instrumental and technical limitations along with the over-simplification of physiological and optical processes affecting their correlations. As an example of an over-simplified physiological process, we demonstrated that leaf PAR absorption, a parameter often over-simplified to be constant or mainly dependent on chlorophyll concentration, can also be affected by processes, such as optical dynamics of epicuticular waxes, which are commonly ignored. Ignoring the role of physiological processes on LOPs can thus lead to unrealistic estimates of photosynthetic dynamics. Aware of the fact that making accurate leaf PAR absorption measurements is difficult in leaves with contrasting size and morphology from that of a broadleaf (e.g. needle-like leaves), this thesis presents a new methodology that allows the direct estimation of PAR absorption. Thus it reduces instrumental and technical constraints associated with the commonly used methodology of computing leaf PAR absorption from leaf reflectance and transmittance spectra, which is error-prone for needle-like leaves. This methodology reduces the methodological constraints on the correlation between LOPs and the dynamics of photosynthesis.

This thesis also presents spatio-temporal constraints affecting the correlations between LOPs and photosynthetic dynamics. Our interpretation of LOPs is not sophisticated enough to successfully track the complex combination of physiological processes that are behind the dynamics of photosynthesis. As an example of temporal constraints that weaken the correlation between LOPs and photosynthetic dynamics, this thesis shows that zeaxanthin-independent processes enhancing the thermal dissipation of excitation energy were not detected by PRI when their correlation was studied at the seasonal scale. This resulted in the overestimation of LUE by PRI, particularly in the early spring. Finally, as an example of a spatial constraint affecting the correlation between LOPs and photosynthetic dynamics, LOPs have a “baseline” component, which is not physiological, but largely dependent of leaf morphology and, to a lesser extent, on the light environment in which the leaves develop. This “baseline” component has a larger effect on reflectance-based optical parameters than on fluorescence-based optical parameters.

Despite the caveats highlighted, this thesis unquestionably shows the large potential of assessing the dynamics of photosynthesis by non-destructive and non-invasive optical means based on leaf reflectance and fluorescence emissions. By identifying sources of variation in reflectance measurements a first step has been taken towards reducing the levels of uncertainty in these estimates in the near future. Even more accurate characterisations of photosynthetic dynamics will be possible in the future, not only at the leaf level, but also at larger scales. However, this requires associating time- and space-

dependent differences in LOPs with specific morphological and physiological processes, and reducing measurement errors through methodological improvements such as those suggested in this thesis.

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