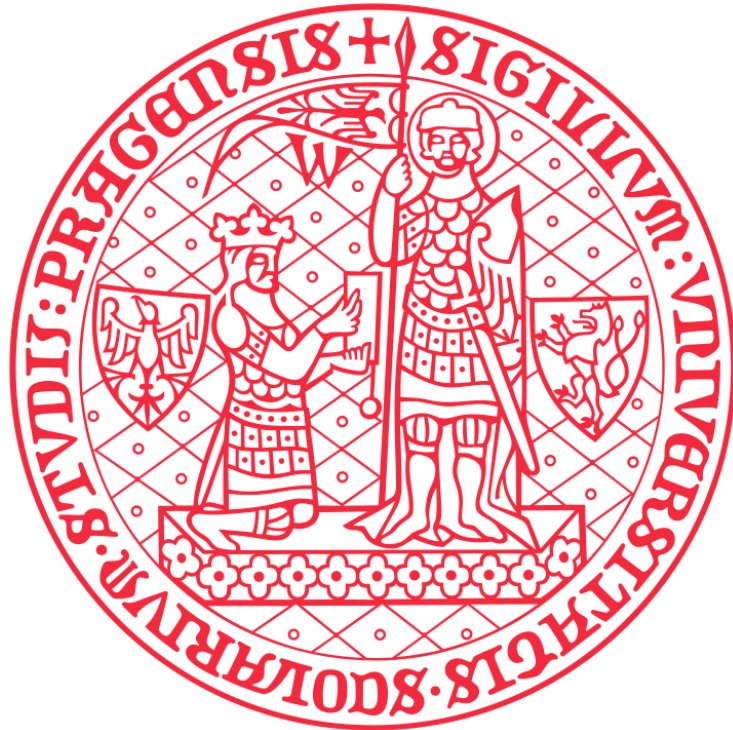


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**Optical properties of the leaf in relation to its anatomical traits  
Optické vlastnosti listu ve vztahu k anatomickým vlastnostem listu**

**Ph.D. Thesis**

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**Prague, 2022**



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## Abstract

Plant functional traits at the leaf level are commonly used to predict ecosystem responses to environmental factors and describe global climate change processes at the ecosystem level. Plant functional traits include both leaf biophysical traits (e.g., photosynthetic pigment content and water content) and structural traits (e.g., leaf thickness and proportion of photosynthetic and non-photosynthetic tissues).

Leaf biophysical and structural traits can be detected either destructively in the laboratory or non-destructively using leaf optical properties. Although estimating chlorophyll content from leaf optical properties is a well-established methodology, the influence of leaf structure and internal anatomy on leaf optical properties has only been thoroughly studied in the last two decades.

The papers included in my thesis and my thesis itself are mostly focused on the study of typical European deciduous trees of temperate and hemiboreal forests with leaves having a dorsiventral structure (i.e., the mesophyll is differentiated into palisade and spongy parenchyma). Furthermore, my thesis includes a study on the effect of leaf surface structural traits on optical properties. In this study, two groups of phylogenetically close herbs with comparable internal leaf structure were used (mutants of *Arabidopsis thaliana* L. and species of the genus *Hieracium*).

In my first paper (Neuwirthová *et al.*, 2017), I considered the optical properties of a single leaf or a stack of five leaves as a simulation of optical properties at the canopy level. In this study, some commonly used vegetation indices used to detect chlorophyll content were found to be strongly influenced by the stack of leaves structural factor, while other vegetation indices were not affected. My contribution in the form of anatomical analysis and its interpretation in the second paper (Lukeš *et al.*, 2020) helped to clarify the effect of internal dorsiventral leaf asymmetry of deciduous trees on their leaf reflectance from different angles of radiation incidence. Upscaling this observation to the canopy level helps to refine the interpretation of optical data at the canopy level during the growing season. Not only the part of the season is important for canopy reflectance, but also the leaf development strategy of a given tree (Neuwirthová *et al.*, 2021a). The formation of young developing leaves was observed throughout the growing season in the studied tree species. Continuous leaf development, therefore, could influence reflectance at the canopy level, not only at the beginning of the growth season but throughout its duration.

The last paper focused on the effect of the surface leaf structure on its optical properties (Neuwirthová *et al.*, 2021b). Apparently, due to the close relationship within the two model groups (*A. thaliana* mutants and several species of the genus *Hieracium* genus) the differences in leaf structural traits were minimal and thus, no effect on the specular and total leaf reflectance was observed. However, we were able to model basic leaf biophysical traits based on leaf optical properties using PLSR, which had not been previously described for the species *A. thaliana*.

The present text of the thesis has been written from a biological perspective to give a more detailed ecophysiological dimension to spectroscopic studies and should contribute to better connecting the fields of plant physiology, anatomy, and laboratory spectroscopy with the spectroscopic methods involved in applied remote sensing.

**Key words:** *Arabidopsis thaliana*; biophysical traits; deciduous trees; diffuse reflectance; dorsiventral leaves; growth season; *Hieracium*; laboratory spectroscopy; leaf epidermis; leaf optical properties; leaf thickness; phenology; planar leaf; specular reflectance



## Abstrakt

K předpovědi reakcí ekosystémů na faktory prostředí se běžně používají funkční znaky rostlin na úrovni listu, popisující projevy globálních změn klimatu na úrovni ekosystémů. Mezi funkční znaky rostlin řadíme jak biofyzikální vlastnosti listu (např. obsah fotosyntetických pigmentů a obsahu vody) tak jeho strukturní vlastnosti (např. tloušťka listu a poměr fotosyntetických a nefotosyntetických pletiv listu).

Biofyzikální a strukturní vlastnosti listu je možné zjišťovat buď destruktivně v laboratoři, nebo nedestruktivně s využitím optických vlastností listu. Ačkoli je odhadování obsahu chlorofylu na základě optických vlastností listů dobře zavedenou metodou, vliv struktury a vnitřní anatomie listů na jejich optické vlastnosti je důkladně studován teprve v posledních dvou dekadách.

Publikace zahrnuté v mé práci a většina práce je věnována evropským opadavým dřevinám, typickým pro temperátní a hemiboreální lesy s listy vykazujícími podobnou dorziventrální strukturu, (tj. mezofyl je diferencován na palisádový a houbovitý parenchym). Dále má disertační práce zahrnuje studii vlivu strukturních znaků povrchu listů dvou skupin bylin na jejich optické vlastnosti. V této studii byly použity dvě skupiny fylogeneticky blízkých bylin se srovnatelnou vnitřní strukturou listů (mutanty *Arabidopsis thaliana* L. a druhy rodu *Hieracium*).

V mém prvním článku (Neuwirthová *et al.*, 2017), jsem se zabývala optickými vlastnostmi jednoho listu ve srovnání s vrstvou pěti listů, jakožto simulace optických vlastností na úrovni koruny. V této studii bylo zjištěno, že některé běžně používané vegetační indexy ke zjišťování obsahu chlorofylu jsou silně ovlivněny strukturním faktorem vrstvy listů, zatímco jiné vegetační indexy ovlivněny nebyly. Můj příspěvek v podobě anatomické analýzy a její interpretace ve studii (Lukeš *et al.*, 2020) pomohl objasnit vliv vnitřní asymetrie listů opadavých stromů na jejich odrazivost z různých úhlů dopadu záření. Přeskálování tohoto pozorování na úroveň koruny přispívá ke zpřesnění interpretace optických dat na úrovni koruny v průběhu sezóny. Ne jen období vegetační sezóny je důležité pro odrazivost koruny, ale také konkrétní strategie vývoje listů daného stromu Neuwirthová *et al.* (2021a). Tvorbu mladých vyvíjejících se listů jsme u studovaných dřevin pozorovali po celou dobu vegetační sezóny. Ukázalo se, že kontinuální vývoj listů by mohl ovlivňovat odrazivost na úrovni koruny nejen na začátku vegetační sezóny, ale po celou dobu jejího trvání.

Poslední studie se zaměřila na vyhodnocení vztahu strukturních vlastností epidermis a optických vlastností listu (Neuwirthová *et al.*, 2021b). Díky blízké příbuznosti v rámci studovaných skupin rostlin (různé linie *A. thaliana* a několik druhů rodu *Hieracium*) a malým rozdíly ve strukturálních znacích jejich listů nebyl zaznamenán vliv na celkovou odrazivost ani zrcadlovou složku odrazivosti. Nicméně se nám podařilo na základě optických vlastností listu pomocí PLSR modelovat základní biofyzikální parametry listů, což dosud nebylo pro druh *A. thaliana* v literatuře popsáno.

Text disertační práce byl napsán z biologické perspektivy, která dává spektroskopickým studiím detailnější ekofyziologický rozměr a měla by přispět k lepšímu propojení oborů fyziologie a anatomie rostlin s laboratorní spektroskopií a spektroskopickými metodami využívanými v aplikovaném dálkovém průzkumu Země.

### Klíčová slova:

*Arabidopsis thaliana*; biofyzikální znaky listu; difúzní odrazivost; dorziventrální list; fenologie; *Hieracium*; laboratorní spektroskopie; opadavé dřeviny; optické vlastnosti listu; planární list; pokožka listu; tloušťka listu; vegetační sezóna; zrcadlová odrazivost





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## Abbreviations

Ames/A = surface area of mesophyll cells per unit leaf area  
Anth = anthocyanins  
BRF = Bidirectional Reflectance Factor  
Car = carotenoids  
Chl = chlorophyll a+b  
CP = contact probe  
CPs= contact probes  
DHRF = Directional-Hemispherical Reflectance Factor  
DHTF = Directional-Hemispherical Transmittance Factor  
EMR = electromagnetic radiation  
EWT = equivalent water thickness  
FOV = field of view  
IS = integrating sphere  
LAI = leaf area index  
LMA = leaf mas per area  
LOP = leaf optical properties  
LT = leaf thickness  
LWC = leaf water content  
N = nitrogen  
NIR = near-infrared region of the electromagnetic spectrum  
PAR = photosynthetic active radiation  
PP = palisade parenchyma  
R/T =reflectance-transmittance ratio  
RE = red edge  
RS = remote sensing  
RTM = radiative transfer model  
RTMs = radiative transfer models  
RWC = relative water content  
SLA = specific leaf area  
SP = spongy parenchyma  
SWIR = short wave infrared region of the electromagnetic spectrum  
TIR = thermal infrared region of the electromagnetic spectrum  
TOC = top-of-canopy  
VI = vegetation index  
VIs = vegetation indices  
VIS = visible part of the electromagnetic spectrum



## **Introduction**

*The importance of remote sensing methods for monitoring the physiological state of vegetation has been growing steadily since the late 1970s, when NASA's Landsat program launched the first satellite to monitor vegetation from Earth orbit (Zemek, 2014). Currently, remote sensing methods play an important role in vegetation monitoring, not only at an ecosystem level, but also at regional and global scales, providing methods for predicting vegetation productivity based on plant functional traits (Niinemets et al., 2015). Plant functional traits are commonly used to predict ecosystem responses and thus contribute to the description of global climate change at the ecosystem level (Liu et al., 2019).*

*It is important to note that net photosynthetic production (NPP), which is currently monitored from orbit, e.g., by the MODIS satellite (Zhao et al., 2011), is influenced by photosynthetic production and the carbon storage capacity of plant biomass (Turner et al., 2006). Based on the use of the MODIS satellite time series in the first decade of this millennium, it was concluded that annual NPP values were significantly affected by droughts brought on by ongoing climate change, leading to stagnation of NPP values rather than the expected increase (Zhao and Running, 2010). Detection of spatiotemporal variation and phenology in a rapid and non-destructive way opens new possibilities for large-scale ecological studies (Chi et al., 2022). By determining the length of the growth season, it is possible to predict climate extremes, e.g., heat waves, floods (Nezval et al., 2020). Thus, the study of the physiological state of vegetation throughout the growth season, i.e., phenological changes, and the ability of vegetation to act as a carbon sink during its phenological development, is of interest not only to plant biologists but also to ecologists, farmers, climatologists, and climate modelers.*

*Earth remote sensing is a powerful tool for monitoring spatial changes and physiological functions of vegetation with respect to canopy structure over time (Noda et al., 2021). Using knowledge of optical properties at the canopy and leaf level, it is possible to model key photosynthetic parameters for entire ecosystems (Croft et al., 2017). This structural variability in vegetation is not only related to the aboveground plant body and canopy architecture but is also manifested at the level of internal leaf structure, which must be considered when interpreting spectral information at the canopy level. An example of such a leaf-level approach is the DLM radiative transfer model, which considers the internal structure of the leaf, as will be demonstrated in one of my papers included in this thesis.*

*To date, many studies have used leaf-level optical properties as a tool to assess plant physiological status. Many of them have focused on the difficult task of improving leaf spectroscopy measurement and processing methodologies to achieve the most accurate non-destructive estimation of leaf biophysical properties according to their optical properties. Although the estimation of chlorophyll content from leaf optical properties is a well-established methodology, the influence of leaf structure and internal anatomy on leaf optical properties has only been thoroughly addressed since 2002 (Sims and Gamon, 2002).*

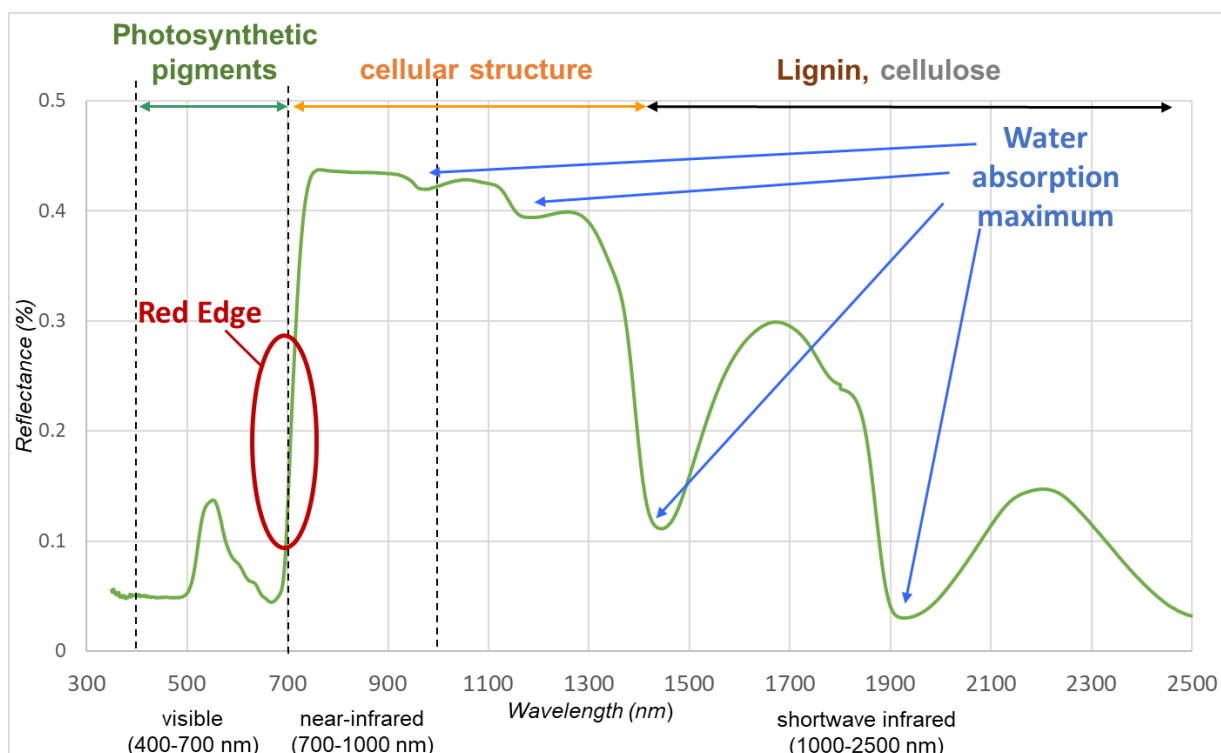
*Advances in the development of remote sensing methods for vegetation monitoring and their accurate interpretation depend on the contribution of plant biologists, making this a multidisciplinary field. My Ph.D. thesis contributes biological understanding to the field of remote sensing by elucidating how the anatomical structure of a leaf and its internal and surface structures affect optical properties with respect to leaf functional traits, often related to leaf phenology.*

# 1 Scientific background

## 1.1 Optical properties at the leaf level

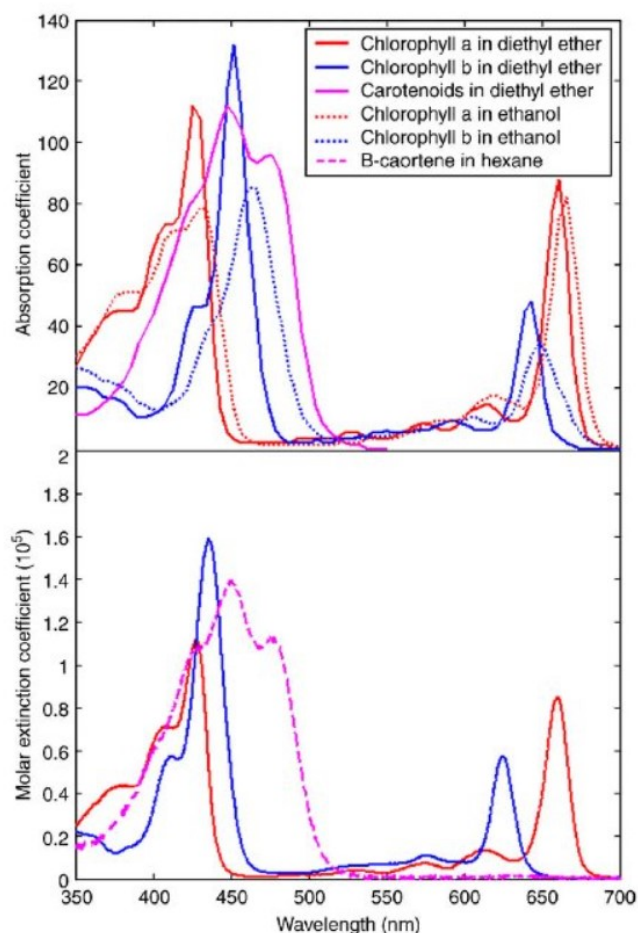
The leaf optical properties (LOP) can be described as the fraction of incident electromagnetic radiation (EMR) flux, that is a) absorbed (absorption); b) reflected (reflectance) or c) transmitted (transmittance) through the leaf. EMR is characterized by the energy of photons at a given wavelength.

The incident radiation that plants use for photosynthesis and other physiological processes – known as photosynthetic active radiation (PAR) (Asrar *et al.*, 1984), is absorbed mainly in the visible part of the spectrum (visible light, VIS), particularly in the red and blue parts (Figure 1), as defined by (Grant, 1997). Specific ranges in the VIS are absorbed by photosynthetically active pigments (chlorophyll a+b and carotenoids; Chl and Car, (Figure 2)), (Sims and Gamon, 2002) and anthocyanins (Anth, (Neill and Gould, 2000)), which are essential non-specific stress indicators providing information on the physiological status of plants (Albrechtová *et al.*, 2017).



**Figure 1** Spectral reflectance curve of vegetation corresponds to reflectance and driving factors determining its course in the range of 350-2500 nm. Red edge corresponds to a steep increase in reflectance on the margin of red part of visible spectrum. Adapted according to: Gates *et al.* (1965).

More detailed information on the absorption maxima of photosynthetic pigments (Chl and Car) and their ranges in different leaf types is given by Ustin *et al.* (2009), (Figure 2). Other chemical compounds in the leaf also have their own absorption maxima at longer wavelengths than VIS, for example: proteins including nitrogen (N) (Berger *et al.*, 2020), as well as lignin and cellulose (Kokaly and Clark, 1999; Serrano *et al.*, 2002) and water (Carter, 1991; Eitel *et al.*, 2006). Absorption features of individual leaf biochemical components of leaves are reviewed in detail by Curran (1989).



**Figure 2**

Upper: Differences in absorption spectra of chlorophyll a, chlorophyll b and  $\beta$ -carotene in diethyl ether and chlorophyll a and b in ethanol. Lower: Molar extinction coefficient of chlorophyll a and b dissolved in diethyl ether and  $\beta$ -carotene dissolved in hexane. Taken from: Ustin *et al.* (2009).

If we know the absorption maximum of a particular biochemical compound in a leaf, we can also detect that compound based on the intensity of light reflected from the leaf in the same wavelength or spectral range (Curran, 1989). To identify and quantify physiological properties of leaves, leaf reflectance has been used since 1929 to compare radiation that has not been absorbed by specific compounds for Chl and Car (Shull, 1929). Not all radiation is absorbed and reflected; just as leaves reflect light, light also passes through leaves – this light quantity is called transmittance. Since the leaf is a complex organ performing a variety of physiological functions, let us call the complex of all chemical elements, biochemical compounds (e.g., Chl, Car, Anth), proteins, structural biopolymers (e.g., lignin, cellulose) of which the leaf is composed, as well as the way the leaf is built in terms of its anatomical structure (saturated by air and water) the “leaf biophysical properties” (Asner, 1998). All these biophysical properties naturally influence LOP together (e.g., Chl content, water content, leaf anatomical structure) (Gates *et al.*, 1965; Asner, 1998) and with the contribution of leaf physiological status (Peñuelas and Filella, 1998) and phenology (Rapaport *et al.*, 2014) this topic presents a broad field for research.

Various mathematical transformations of the vegetation spectral curve have been used to identify and quantify individual leaf traits (Shull, 1929) and these methods are still developing (Verrelst *et al.*, 2019). The vegetation spectral curve is a common way to visualise LOP as a percentage of reflected or transmitted light relative to a white reference standard. The white reference standard is an ideal diffuse Lambertian reflector that is used as a calibration panel, reflecting light perfectly and constantly in all directions (Schaeppman-Strub *et al.*, 2006). Currently,

the white reference standard is typically made of barium sulphate ( $\text{BaSO}_4$ ) with 99% reflectance at all wavelengths sampled by the spectroradiometer (Jacquemoud and Ustin, 2019). The spectral curve shows reflectance or transmittance value at each wavelength in spectral ranges covered by the sensor. The most commonly studied ranges of spectral curve are: visible (VIS; Niglas *et al.*, 2017) through near-infrared region (NIR; (Slaton *et al.*, 2001)) to short wave infrared 1 and 2 (SWIR 1,2; (Cavender-Bares *et al.*, 2016)) with occasional studies focusing on the thermal infrared region (TIR; (Gerber *et al.*, 2011)).

Since the 1980s, remote sensing (RS) studies have been used (Schowengerdt, 2006) to detect the distribution and diversity of plants (Wang and Gamon, 2019), as well as their physiological status and productivity (Peñuelas and Filella, 1998). From the 1980s onwards, quantitative remote sensing emerged, the products of which must be validated by ground truth data collection, including leaf-level reflectance data. (Buschmann and Nagel, 1993). Reflectance measurements at the leaf level are a rapid, inexpensive, and non-destructive way to assess in situ leaf chlorophyll content, one of the most important indicative parameters of physiological status (Gitelson *et al.*, 2003; Lu *et al.*, 2015), which is closely related to plant stress (e.g., (Campbell *et al.*, 2004) and leaf phenological status (Junker and Ensminger, 2016).

My Ph.D. thesis focuses on contributing to the understanding of how leaf anatomy affects LOP and connecting leaf anatomical changes to LOP and leaf phenology. In the following chapters I will discuss the basis for interpreting LOP relating to leaf chemistry and leaf structure.

## **1.2 Leaf traits affecting LOP in selected spectral ranges**

The following text will include information about leaf reflectance in particular spectral regions, (VIS, Red Edge, NIR, SWIR) which serves as the traditional approach in well-established spectroscopic literature (Gates *et al.*, 1965) as well as in the TIR spectral band as a direction of field of interest for the future. The aim of the subsequent paragraphs is to bring proper information about a specific part of the spectral curve in a specific spectral region and response of the wavelengths to leaf biophysical leaf traits.

### *1.2.1 Response of the reflectance in visible part of the region is driven by photosynthetic pigments*

In spectroscopy studies, the VIS is considered to be the region where the LOP responds primarily on pigment content, in the regions from 300-700 nm (Gates *et al.*, 1965), and 400-700 nm (Neuwirthová *et al.*, 2017) (Figure 1). Typically, the leaf reflectance curve in VIS can be described by a local minimum in the blue region (450-500 nm), a maximum in the green (540-560 nm) and then another minimum in the red (660-680 nm) (Clark and Lister, 1975). The decrease in reflectance corresponds to the absorption of chlorophyll: the maximum absorption of Chl *a* and Chl *b* is between 590-660 nm and carotenoids have absorption maxima at 425, 450 and 480 nm (Gitelson and Merzlyak, 1994). Some studies have shown that reflectance in VIS can be affected by leaf surface structure (Shull, 1929; Buschmann *et al.*, 2012) and water content (Carter, 1991). Higher trichome density has been found to cause greater total reflectance in the VIS (Billings and Morris, 1951). How leaf surface affects LOP will be discussed in Chapter 1.4.4



### 1.2.2 *Red edge and its inflection point of vegetation spectral curve responds to stress state in plants*

The sharp increase in reflectance between VIS and NIR is called the “Red Edge” (RE) and is usually defined by a wavelength range of 680-750 nm. The RE is directly related to the Chl content of green leaves (Sims and Gamon, 2002). Specifically, the position of the inflection point of the spectral curve (the extreme of the first derivative of the spectral curve at given wavelengths) serves as an indicator of plant stress (Gitelson *et al.*, 1996). A shift of the RE position towards lower wavelengths is called a “blue shift” (Rock *et al.*, 1988) and corresponds to worsened physiological status, whereas its shift towards longer wavelength is called a “red shift” and corresponds to an improved plant physiological status (Filella and Penuelas, 1994).

### 1.2.3 *Leaf internal structure is manifested in near infra-red region leaf optical properties*

It is now generally accepted that NIR (750-1350 nm) reflectance (Gates *et al.*, 1965; Eitel *et al.*, 2006) is primarily influenced by the internal structure of the leaves (Slaton *et al.*, 2001; Buschmann *et al.*, 2012) and the water content in leaf tissue, manifesting absorption maxima at approximately 970 and 1200 nm (Sims and Gamon, 2003; Zhao *et al.*, 2016) (Figure 1). Leaf structure, and thus LOP in the NIR, depends on the leaf developmental stages (Dengler *et al.*, 1975; Rapaport *et al.*, 2014) during the season (Yang *et al.*, 2016), i.e., leaf phenology, and position in the canopy (sunlit or shaded leaves) (Hanba *et al.*, 2002). At the canopy level, reflectance in the NIR is also often correlated with leaf area index (LAI) (Blackburn, 1999; Fang *et al.*, 2019). At the leaf level, LAI has been approximated by leaf stacking. This issue is discussed in the first article of this thesis (Neuwirthová *et al.*, 2017) and mentioned in Chapter 4.4.

### 1.2.4 *Structural compounds reflect radiation in short wave infra-red region*

The spectral curve of vegetation, after its course in VIS and NIR, continues in mid-infrared region (1350-2500 nm) (Gates *et al.*, 1965; Hunt and Rock, 1989), currently referred to more as the shortwave infrared region (SWIR) (Serrano *et al.*, 2000) (Figure 1). This region is sometimes further subdivided into the SWIR1 (1500-1800 nm) and SWIR2 (2000-2400) regions (Cavender-Bares *et al.*, 2016; Neuwirthová *et al.*, 2017). The reflectance of leaves in the SWIR region, similarly to the NIR, is largely dependent on the water content of the leaves (i.e., water absorption in the 1450, 1940 and 2500 nm (Carter, 1991; Zhao *et al.*, 2016). Lignin, an important polyphenolic compound found in plant cell walls along with cellulose, also models the SWIR region with its specific absorption properties (Daughtry, 2001; Serrano *et al.*, 2002; Nagler *et al.*, 2003).

### 1.2.5 *Thermal radiation is affected by leaf biophysical and structural traits*

Compared to reflectance in the previously described spectral regions of VIS, NIR and SWIR, emission of thermal radiation by a leaf; i.e., thermal infrared region (TIR; 8-14  $\mu\text{m}$ ) (Gerber *et al.*, 2011) is not a common methodology in laboratory spectroscopy. However, emission or reflectance in the TIR can also be associated with physical changes in leaves, for example, water, lignin or cellulose contents and leaf area or plant stress (Buitrago Acevedo *et al.*, 2017). A recent study concluded that, like VIS, NIR and SWIR, reflectance in TIR is dynamic over time during leaf development (Richardson *et al.*, 2021). Based on the conclusions of the study by Gerber *et al.* (2011), the reflectance in TIR is also influenced by leaf surface properties, as discussed in our recent paper (Neuwirthová *et al.*, 2021b). It is meaningful to test how TIR reflectance responds to biophysical

properties and physiological status at the leaf level. We collaborated with colleagues at the Global Change Research Institute on these measurements and conducted a joint preliminary study of selected deciduous leaf species. For this, we used a Mid-IR IntegratIR™ spectroradiometer from PIKE Technologies™ equipped with an integrating sphere for to measure the infrared transmission Spectrometer was further attached to a spectroscopy Mid-IR Nicolet 380, with a resolution from  $0.07\text{ cm}^{-1}$  in 2000 nm to 60 nm near the 18000 nm. The data are not yet processed and are not the subject of this study, but we anticipate that this analysis could be used in the future to measure ground truth for TIR sensors commonly used in RS studies.

### **1.3 Application of vegetation spectral properties for vegetation and ecosystem monitoring**

RS allows non-destructive assessment of stress, physiological status or productivity of vegetation over a large area (Schowengerdt, 2006). In terms of plant ecophysiology, the most important RS applications are those related to environmental assessment, global change (deforestation, global warming, drought), agriculture management (yield prediction, soil erosion) and many others (Schowengerdt, 2006), such as the carbon cycle characteristics – i.e., determining the sink or source strength of an ecosystem (Xiao *et al.*, 2019).

For decades, RS has been used to detect water stress (Hunt and Rock, 1989), gross and net primary production (Turner *et al.*, 2006), the role of terrestrial ecosystems in the global carbon cycle (Zhao and Running, 2010; Hinojo-Hinojo and Goulden, 2020), the impact of anthropogenic air pollution on forest cover and health (Kupková *et al.*, 2018), forest disturbance (Švik *et al.*, 2020), leaf physiological status (Peñuelas and Filella, 1998), and nitrogen content detection (Serrano *et al.*, 2000), often with the advantage of high spatiotemporal resolution of satellite systems (Mišurec *et al.*, 2016). Spectral reflectance data are also used for indirect detection of plant diseases, for example, the study by Mahlein *et al.* (2013) focused on the detection *Cercospora* leaf spot, sugar beet rust and sugar beet powdery mildew.

RS methods can be active, or passive based on the technology used. However, active remote sensing technologies that require a source of energy, (e.g., the laser in light detection and ranging (LiDAR) systems (Dubayah and Drake, 2000)), are not focus of this thesis. Passive RS is typically based on the detection and processing of EMR that is reflected from vegetation, its canopy architecture, understory and finally ground (Jensen, 2009) and uses solar radiation as a light source. Observed reflectance depends on both the direction of incoming light (Schowengerdt, 2006; Lukeš *et al.*, 2020), and the observation geometry, and is driven by wavelength and biophysical properties of the Earth's surface (Jensen, 2009).

Remote sensing studies are driven by the demand for applied research that makes use of advanced remote sensing technologies, but with the limitations outlined below:

- Measurements of Earth's surface reflectance could yield erroneous interpretations due to a combination of different factors, particularly as information is affected by the current state of the atmosphere at the location and time of data acquisition. High-quality atmospheric corrections are critically needed, particularly in tropical rainforests where aerosols and water vapour concentrations are a source of uncertainty in interpretation of plant physiological status (Hilker *et al.*, 2012), but they are of course also needed in other ecosystems.
- RS interpretation is complicated by other factors such as the spectral mixing of the respective signals of plants and soils (Martinez *et al.*, 2015), plant canopy structure (Eitel *et al.*, 2006), and different leaf angle position (Jay *et al.*, 2016) especially in NIR and SWIR spectral regions (Neuwirthová *et al.*, 2017). The effects of the leaf angle geometry on the canopy reflectance is discussed in the article Lukeš *et al.* (2020) in the present thesis.

The RS observations are conducted on different platforms corresponding to different hierarchical levels:

- i) Satellite sensors combine the advantage of sensing large areas and the capability to acquire time series at intervals of days to weeks. However, they typically have the disadvantage of low spatial resolution and limited spectral range. In addition, the thick layer of atmosphere between the satellite sensor and the Earth's surface lead to the need for various atmospheric corrections. Some areas, such as tropical rainforests, are often cloudy and taking satellite images with passive RS sensors could thus be a difficult task.
- ii) Airborne sensors are flexible and allow the acquisition of data at very high spatial and spectral resolution, but they are very dependent on atmospheric conditions, which limits their use. Additional disadvantages include the limited area that can be covered by aircraft and the impossibility of working with dense time series. Finally, these data are expensive to acquire.
- iii) Unmanned Automated Vehicles (UAVs) carrying spectral sensors have a relatively high acquisition cost (but still lower than airborne RS) and thus offer a suitable alternative to airborne RS due to their greater flexibility in data acquisition. The growing popularity of remote sensing of vegetation using UAVs is particularly evident in phenology studies (D'Odorico *et al.*, 2020), sensing physiological status in orchards (Vanbrabant *et al.*, 2019) and other agroecosystems (Hunt *et al.*, 2005) reviewed by (Zhang and Kovacs, 2012).

Compared to spectral data obtained at higher hierarchical levels, leaf-level data provide information only on the biophysical properties of a given leaf, without aerosols in the atmosphere, canopy structure or soil background. Thus, the basic research on the leaf level optical properties forms the background for interpreting data obtained at the canopy level. Optical properties at the canopy level need to be linked to the leaf level through modelling using radiative transfer models (RTMs, Chapter 1.5.4, Figure 4).

## 1.4 Leaf level measuring methods of leaf optical properties

Leaf optical properties are closely related not only to the chemical composition of foliage but also to their internal anatomy (see Figure 1 in (Lukeš *et al.*, 2020) and Figure 1 in (Neuwirthová *et al.*, 2021a)) and serve as validation data for radiative transfer modelling, mentioned in Chapter 1.5.4. LOP are measured in laboratory conditions at the leaf level by a spectroradiometer, usually in the range 300-2500 nm. The spectral reflectance curve- LOP- obtained in this way provides insights into the biochemical, biophysical properties and leaf physiological status more easily than the determination of leaf conditions by analytical methods.

### 1.4.1 Contact measuring by spectroradiometer equipped with its own source of light

In the majority of measurement setups, spectroradiometers are used for contact measurements (with own source of light) equipped with: a) an integrating sphere (IS) (Gates *et al.*, 1965), and b) a contact probe (CP) (Potůčková *et al.*, 2016). Only a couple of studies have equipped a spectroradiometer with a leaf clip (Dillen *et al.*, 2012; Cavender-Bares *et al.*, 2016), which allows non-destructive in-situ measurements on attached leaves. The advantage of this setup, in contrast to the previous ones, is that such measurements can be repeated over time (Rapaport *et al.*, 2014) on the same plant. This last setup will not be discussed further.

a) IS is a well-established tool (see Figure 3 in (Neuwirthová *et al.*, 2021b)) for obtaining leaf optical properties and retrieving various leaf biochemical traits at the leaf level (Gates *et al.*, 1965; Gausman and Allen, 1973; Croft *et al.*, 2014; Lukeš *et al.*, 2020). The IS is designed with a sample port, or the possibility to place a leaf sample in the IS and adjust measurement setup for reflectance and transmittance (Olascoaga *et al.*, 2016; Potůčková *et al.*, 2016; Hovi *et al.*, 2017) or diffuse reflectance if the IS type allows it (Neuwirthová *et al.*, 2021b), this type of measurement is further discussed in Chapter 1.4.4. The use of IS is more time- and skill-demanding, but nonetheless more accurate than CP measurements. The measured quantity – hemispherically integrated reflectance and transmittance is also the only LOP comparable between datasets, due to the removal of the influence of observation geometry. These measurements are thus suitable for the creation of spectral databases.

b) The previously mentioned complementary device for spectroradiometers is the CP (Eitel *et al.*, 2006), also appropriate for non-destructive measurements of plant physiological status at the leaf level in field studies. In contrast to IS, which can provide both reflectance and transmittance, CP is designed only for reflectance measurements, however, the use of CP for transmittance is discussed in Hovi *et al.* (2017). The CP was designed to measure larger dorsiventral leaves covering the entire field of view (FOV), with the advantages of faster, more convenient measurements, and simpler data processing than the IS. Contact probes (CPs) have been used for contact measurement and detection of chlorophyll content (Lu *et al.*, 2015), leaf nitrogen content (Wang *et al.*, 2017), or water status in deciduous trees (Eitel *et al.*, 2006) and leaf phenology in dorsiventral leaves (Rapaport *et al.*, 2014).

CPs are mainly used on dorsiventral leaves of deciduous trees, crops, and occasionally needles, primarily for ecophysiological studies. Based on our research, thin, delicate leaves, such as those of *A. thaliana*, are not a common material for contact measurement, except for Martinez *et al.*, (2015), who measured physiological status of *A. thaliana* using a CP, as will be discussed in Chapter 4.1.5

Recent studies have also have focused on comparing different measuring devices (Hovi *et al.*, 2017), including our study (Potůčková *et al.*, 2016), or different measuring setups of dorsiventral leaves (Datt, 1999), also included as part of our study (Neuwirthová *et al.*, 2017), and needles on twigs or cut twigs (Einzmann *et al.*, 2014).

#### 1.4.2 *Non-contact measuring by spectroradiometer with an additional source of light*

Another measurement method can be non-contact measurements from nadir with an additional light source (Rock *et al.*, 1994; Datt, 1999; Campbell *et al.*, 2004; Eitel *et al.*, 2006; Lhotáková *et al.*, 2007; Castro and Sanchez-Azofeifa, 2008; Albrechtová *et al.*, 2008; Lu and Peng, 2015). Using solar radiation as a light source is the simplest way to non-destructively measure optical properties of vegetation at the canopy level in seedlings (Merlier *et al.*, 2015), crops (Pimstein *et al.*, 2011), and orchards (Wang *et al.*, 2017).

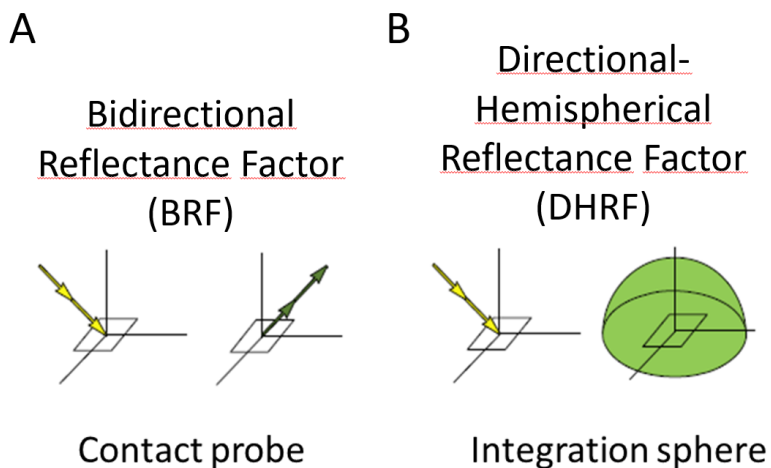
#### 1.4.3 *Terminology of the selected reflectance quantities*

Although this thesis is not focused on remote sensing studies of vegetation at the canopy level, but on the acquisition of the spectral data at the leaf level using a spectroradiometer equipped with CP or IS, I use the general terminology framework developed for RS studies. The basic concepts, definitions, and terminology of reflectance quantities are given by (Nicodemus *et al.*, 1977) and the angular characteristics of reflectance distributions are summarised by Schaepman-Strub *et al.* (2006). For the purposes of this thesis, I will provide a definition of reflectance factor, which is used to define a reflectance quantity based on the terminology given by Schaepman-Strub *et al.* (2006): “The reflectance factor is the ratio of the radiant flux reflected by surface to that reflected into the same reflected beam geometry and wavelength range by ideal and diffuse (Lambertian) standard surface.” (Schaepman-Strub *et al.*, 2006).

In the present thesis, I used two reflectance quantities to obtain spectral data (Schaepman-Strub *et al.*, 2006). Based on the equipment used to measure spectra in the presented papers, two selected reflectance quantities were examined more closely: 1) Bidirectional Reflectance Factor (BRF), measured by a CP (Neuwirthová *et al.*, 2017); and 2) Directional-Hemispherical Reflectance Factor (DHRF)/Directional-Hemispherical Transmittance Factor (DHTF) measured by an IS (Lukeš *et al.*, 2020; Neuwirthová *et al.*, 2021a) (see Figure 3).

1) In reflectance measurements using CPs, the flux of a parallel beam of incident light from one direction from a light source inside the CP into another direction is recorded, where the optical cable is also fixed inside the CP, which corresponds to BRF (Figure 3A). However, the orientation of the leaf may affect the measurement, particularly in leaves with isotopically oriented epidermal cells. The preferential orientation of epidermal cells (e.g., in monocot grasses) may affect the directionality of the light beam (Comar *et al.*, 2012).

2) The advantage of an IS, where DHRF or DHTF are measured, is the integration of reflected light from the entire hemispherical surface, i.e., less dependence on the orientation of cells on the leaf surface (Figure 3B). An approximation of the DHRF from BRF is described in Comar *et al.* (2012).



**Figure 3**

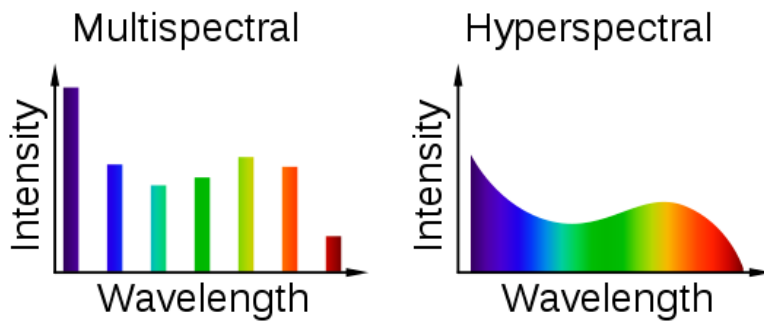
Relation of incoming and reflected radiance terminology used to describe two reflectance quantities. A: Bidirectional Reflectance Factor (BRF), measured by CP, and B: Directional Hemispherical Reflectance Factor (DHRF) measured by IS; corresponds to nomenclature of Nicodemus *et al.* (1997). Taken from Schaepman-Strub *et al.* (2006).

#### 1.4.4 Improvement of Chl content detection by surface reflectance removal

Leaf reflectance can be measured in many ways in addition to BRF and DHRF described above in (Figure 3) (see Schaepman-Strub *et al.*, 2006). The earlier assumption that the leaf is a Lambertian reflector has been rejected by many studies, including Gates *et al.* (1965) and Grant (1987). Nevertheless, there is an amount of the light that is reflected from the leaf surface rather than by the leaf internal structure and biophysical traits. Leaf surface reflectance is controlled by two mechanisms: 1) specular (mirror-like) reflection, in which the angles of incidence and reflection of light are equal (Vanderbilt *et al.*, 1985), and 2) surface particle scattering, which depends on surface roughness and is likely neglected in most plant studies (Grant *et al.*, 1993). Specular reflectance brings difficulties in estimating chlorophyll content in plants with extremely high surface reflectance (Bousquet *et al.*, 2005). In the paper presented in this thesis (Neuwirthová *et al.*, 2021b) we attempted to investigate the effect of leaf surface anatomical structure (trichome size and density) on the specular component of leaf reflectance. We hypothesized that knowledge on the influence of surface epidermal structures on specular reflectance may be useful for improving RTMs using structural traits (Qiu *et al.*, 2019), (Chapter 1.5.4).

### 1.5 Spectral data evaluation, retrieval methods

Hyperspectral sensors operate in hundreds or thousands of contiguous narrow bands, similarly to data acquired by spectroradiometers (Milton, 1987; Potůčková *et al.*, 2016). In contrast to hyperspectral sensors - commonly used today, there are also multispectral sensors, which sense only specific ranges, in the tens to hundreds of nanometres in a few spectral channels (Falcioni *et al.*, 2020). Currently, most laboratory and airborne sensors (Chapter 1.3) are hyperspectral, both at the leaf and canopy level (e.g., APEX, (Schaepman *et al.*, 2015)). However, multispectral sensors are not outdated: good examples are the TM (Sobrino *et al.*, 2004) and OLI sensors onboard the Landsat, or the MSI sensor data on a pair of Sentinel-2 satellites (Claverie *et al.*, 2018). Affordable UAV systems can also be equipped with multispectral sensors. However, further multispectral data processing will not be dealt in the present thesis.



**Figure 4**

Resolution of the multispectral data, acquired by the e.g., Landsat system in a few ranges, compared to hyperspectral data with contiguous narrow bands.

Source:

[https://commons.wikimedia.org/wiki/File:Spectral\\_sampling\\_RGB\\_multispectral\\_hyperspectral\\_imaging.svg](https://commons.wikimedia.org/wiki/File:Spectral_sampling_RGB_multispectral_hyperspectral_imaging.svg); 2021-08-31 16:09:24

The nature of hyperspectral data obtained by a spectroradiometer with a sampling resolution of 1 nm is multicollinear, meaning that the spectral reflectance or transmittance values are correlated and not independent. This multicollinearity is due to redundant information in the hyperspectral data. To detect and exclude redundant bands, dimensionality must be reduced using statistical methods or empirical models (Verrelst *et al.*, 2019).

For clarity, approaches to spectral data processing are divided into four categories according to computing complexity (also see, Li *et al.*, (2019)). The first three approaches (i-iii) are based on empirical models, and the last category is based on physical models (iv):

(i) Average reflectance in the spectral intervals or single wavelength, which could also serve as a basis for the construction of vegetation indices (VIs), see following Chapter 1.5.1 and website Index Database<sup>1</sup>.

(ii): Multivariate statistical analysis (Li *et al.*, 2019) (e.g., principal component analysis (PCA)); various regression methods (e.g., partial least square regression (PLSR) (Chavana-Bryant *et al.*, 2019; Wu *et al.*, 2019; Lhotáková *et al.*, 2021)); spectral transformation (e.g., continuum removal (Kokaly and Clark, 1999)); or nonlinear non-parametric methods. Retrieval methods based on regression, assuming training and validation the dataset, are well reviewed by Verrelst *et al.*, (2019).

(iii) Machine learning: “Non-parametric models are adjusted to predict a variable of interest using a training dataset of input-output data pairs, which come from concurrent measurements of the parameter and the corresponding radiometric observation.”<sup>2</sup> (Féret *et al.*, 2019; Yang *et al.*, 2020).

(iv): Methods developed for interpretation of spectral data (sensitivity analysis or product retrieval) using RTMs (Verrelst *et al.*, 2019).

In the following paragraphs, I will discuss the methods for evaluating spectral curves that I have used in the work presented in this thesis in more detail.

### 1.5.1 Vegetation indices

The classic and most simple way of evaluating spectral curves are vegetation indices (VIs) (see the website Index Database). VIs were created to estimate various vegetation parameters depending on the input spectral channels. One of the most popular VI, Normalized Difference Vegetation Index (NDVI), was developed for evaluating vegetation from Landsat multispectral data

<sup>1</sup> <https://www.indexdatabase.de/>; 2021-08-31 16:09:24

<sup>2</sup> Cited word-for-word from: <https://artmotoolbox.com/79-retrieval/3-machine-learning-regression-algorithms-toolbox.html>; 2021-08-31 16:12:21

in 1974 by (Rouse *et al.*, 1974). VIs are developed taking into account the dimensionality of the data and level of the acquisition (when multispectral data were not multicollinear but separated within a few intervals). Some VIs use broad band spectral channels of multispectral data, which are not able to track the narrow absorption properties of chemical compounds (e.g., chlorophyll, carotenoids, xanthophylls, etc.) and are suitable only for studying the vegetation properties that are manifested by spectral changes over a wide range of wavelengths (e.g., total biomass, and to some extent water content). VIs based on hyperspectral data can take advantage of the narrow spectral properties of a particular compound and target their design on a specific wavelength. These VIs enable the comparison of hyperspectral data at different levels (e.g., leaf and canopy level (Main *et al.*, 2011; Croft *et al.*, 2014)).

Authors Lu *et al.* (2015) classified VIs into five categories according to their mathematical expression:

**Table 1** Examples of vegetation indices and their mathematical expression. *R*=reflectance. Taken from Lu *et al.* (2015).

single-band reflectance or single-difference index between the reflectance of two bands	$RX; 1/RX-1/RY$	(e.g., $R680$ or $1/R515-1/R550$ )	(Blackburn, 1998; Yi <i>et al.</i> , 2014)
simple-ratio index (ratio of reflectance in two wavelengths / wavebands)	$RX/RY$	(e.g., $R672/R550$ )	(Datt, 1998)
normalized difference of reflectance	$(RX-RY)/(RX+RY)$	(e.g., PSNDb: $(R800-R650)/(R800+R650)$ )	(Blackburn, 1998)
indices using reflectance derivatives	$DRX$	(e.g., $D730$ )	(Richardson <i>et al.</i> , 2002)
other forms of indices	$RX/(RY+RZ)$	(e.g., $R705/(R717+R491)$ )	(Tian <i>et al.</i> , 2011)

VIs are usually based on highlighting the contrast between the absorption maximum of the target biophysical trait and the spectral region that is unaffected by this trait. An example design for a chlorophyll VI is that one wavelength is selected from the VIS spectral region, where leaf reflectance is predominantly governed by pigment content, and the second wavelength from the NIR, which is not affected by pigment content. However, some VIs also use NIR reflectance, including the red edge inflection point (Hallik *et al.*, 2019), and are thought to be governed by leaf structure (Slaton *et al.*, 2001). When the NIR region is involved, VIs become insensitive to leaf structure and predict Chl better than VI based on VIS alone (Neuwirthová *et al.*, 2017). As mentioned in Chapter 1.2.3, the internal leaf structure strongly influences the LOP and thus the non-destructive spectral quantification of leaf biochemical compounds. Therefore, it is desirable to develop an algorithm that minimizes sensitivity to leaf structure (Sims and Gamon, 2002; Gitelson *et al.*, 2003).

Aside from Chl content, other biophysical traits with unique spectral features can be estimated using VIs, as summarised by Curran (1989). However, for some substances, such as lignin,



the NDLI (Normalized Difference Lignin Index) only worked after accounting for the green vegetation structure (Serrano *et al.*, 2002), despite known absorption peaks.

#### 1.5.2 *Reduction of the dimensionality spectral data*

Principal Component Analysis (PCA) is a widely used method for reducing dimensionality of collinear spectral data (Esbensen *et al.*, 2018; Li *et al.*, 2020), where the entire spectrum is processed and only the components that explain the largest amount of variability are used for the further analysis (Zhao *et al.*, 2016). PCA is commonly used in multivariate statistics, such as Redundancy Analysis (RDA) or Linear Discriminant Analysis (LDA). The application of multivariate statistics in spectral data is thoroughly explained in Falcioni *et al.* (2020) based on Unscrambler software. This approach was used in my study (Neuwirthová *et al.*, 2021a), and it is also possible to combine PCA with subsequent evaluation of VIs, as we performed in Neuwirthová *et al.* (2021a).

#### 1.5.3 *PLSR Modelling leaf traits by nonparametric methods*

Partial least square regression (PLSR) belongs to linear nonparametric methods (Verrelst *et al.*, 2019) and uses full spectral range rather than a band-by-band approach (Asner *et al.*, 2011). “The basic idea behind PLSR is reducing a large number of reflectance or their derivatives to a few principal components, and making regression using several selected components.” (Yang *et al.*, 2020). Compared to PCA, PLSR incorporates all wavelengths into a linear model and finds a single equation, which is then used for cross-validation, for example, through a “leave-one-out” method (Yi *et al.*, 2014; Esbensen *et al.*, 2018). Methods using full spectral information have become more popular with increasing computational power and the development of software tools. PLSR is a typical retrieval method used for biophysical parameters estimation and extrapolation from spectral data, which enables to use the trained model for an independent spectral dataset (Esbensen *et al.*, 2018). In recent studies, PLSR has been shown to be an accurate and robust method for estimating plant physiological state compared to other regression methods (Siegmann and Jarmer, 2015), sometimes counted among machine learning methods (Yang *et al.*, 2020).

#### 1.5.4 *Radiative transfer modelling - validation leaf/vegetation spectral data by their biophysical parameters*

Physically based RTMs use several input biophysical and structural parameters to simulate the spectral reflectance of leaves or whole vegetation canopies. RTMs are parametrized in such a way that the spectra simulated by the RTM are as close as possible to the physically measured spectra of the leaf or canopy, i.e., the observed reality of leaves or vegetation canopy with given biophysical properties.

RTMs have been developed for different hierarchical levels: leaf level (e.g., PROSPECT (Jacquemoud and Baret, 1990), DLM (Stuckens *et al.*, 2009), LIBERTY (Dawson *et al.*, 1998)) or canopy level (e.g., DART- discrete anisotropic RTM (Gastellu-Etchegorry *et al.*, 2004), SAIL (Verhoef, 1984)).

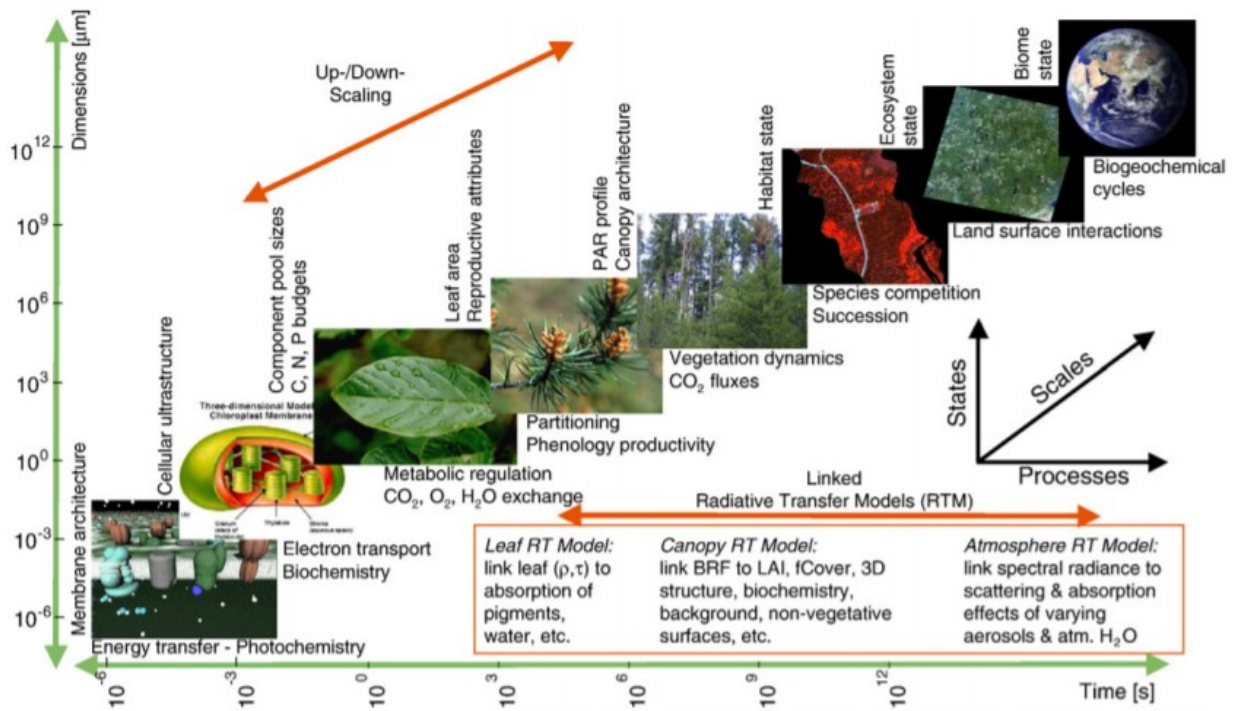
The most common radiative transfer model at the leaf level is generalised “plate model”, the so called PROSPECT (Jacquemoud and Baret, 1990). In this model, leaf thickness (LT) and internal structure are simplified by the parameter “number of the layers” - N. Only three input parameters enter the PROSPECT model: Chl content, water content, and N. Many authors have focused on

improving and extending the basic PROSPECT model, resulting in the following models: PROSPECT 4, which includes four inputs: Chl and water content, N, plus dry matter content; and PROSPECT 5 which is extended to include a fifth parameter: carotenoid content (Feret *et al.*, 2008).

As I mentioned in Chapter 1.2.3, leaf structure plays an important role in LOP. Ma *et al.* (2007) presented a radiative transfer model called QSPECT, where the leaf consists of four layers corresponding to the upper and lower epidermis, the palisade, and the spongy parenchyma. Although some RTMs accounted for more realistic leaf structure (e.g., QSPECT), the dorsiventral asymmetry was largely neglected in the RTMs described above. A later study (Stuckens *et al.*, 2009) extended the PROSPECT model to account for the leaf surface and dorsiventral nature of the planar leaves by including several structural parameters, namely: palisade parenchyma (PP) - facing the adaxial side of the leaves, and spongy parenchyma (SP), with extensive air space volume - facing the abaxial leaf side, and the PP/SP ratio. The model is called the Dorsiventral Leaf Model (DLM) and was used for the sensitivity analysis of simulated and measured LOP of dorsiventral leaves in our paper (Lukeš *et al.*, 2020) presented in the thesis.

If the RTM is run in forward mode, the spectral curve of the leaf, or whole canopy is generated based on the input biophysical and structural parameters. To obtain the biophysical parameters from empirically measured spectra, backward run (inversion of the model) is used; reviewed by Verrelst *et al.*, (2019). The RTMs at the leaf level are provided by physically measured spectra as an input and leaf biophysical traits are estimated as an output. The estimated biophysical traits can then be validated by comparison with measured leaf biophysical traits. With this inversion approach, it is possible to upscale spectral data to higher hierarchical levels and estimate biophysical parameters at those hierarchical levels without the need for extensive ground truth data collection.

The combination of RTMs operating at different hierarchical levels is possible and allows upscaling or downscaling of reflectance and retrieval of biophysical and structural traits at various levels. For example, a study by Jacquemoud *et al.* (2009) combined the leaf-level PROSPECT model and the canopy-level SAIL model to develop the PROSAIL model, which accounts for leaf biophysical properties along with canopy architecture and the directionality of incident and reflected light at the canopy level. In our case (Lukeš *et al.*, 2020), we coupled the DLM model (incorporating asymmetry of internal leaf structure) with the DART model, which incorporated information at the canopy level about the angular distribution of leaves and the overall structure of the studied forest stands. Linking these two models together allowed us to evaluate the effect of internal leaf asymmetry on the top-of-canopy (TOC) reflectance. The observation that neglecting leaf asymmetry can introduce a relative difference of 20% in TOC reflectance can remarkably affect the interpretations of RS studies, especially in deciduous trees with dorsiventral internal leaf structure (Lukeš *et al.*, 2020).



**Figure 5** Coupled states, processes and scales ranging from cellular architecture to global biogeochemical cycles. The contribution of linked radiative transfer models in down-and upscaling ranges from leaves to biomes. Taken from Schaepman et al. (2009).

## 2 Aims

The aims of my research were to expand knowledge of LOP measured by different methods and their use in phenological studies, and to improve the relationship between leaf structural traits and leaf optical properties.

The first objective was more methodological, A) and was discussed under two aims **1)** the influence of optical property measurement methodology on LOP; and **2)** the relationship between structural and optical properties of leaves.

The second objective of my research presented different applications of LOP in relation to structural traits, B) the use of LOP as influenced by leaf structure to characterize the phenological phase of the leaf during the growing season, which was studied under aims **3,4)**.

- A) To contribute to knowledge on the influence of leaf structural traits in different spectral regions:
- 1) To compare the leaf spectral reflectance on a single leaf and a stack of leaves in the VIS, NIR, and SWIR spectral ranges, and to test if and how selected vegetation indices differ in these two measurement setups.**
  - 2) To quantify the effects of selected quantitative epidermal traits (trichome density and length, and adaxial epidermis thickness) on specular reflectance in VIS and NIR spectral regions.**
- B) To use knowledge on relation of leaf structural traits with LOP to characterize dynamics of leaf chemical composition during its phenological development:
- 3) To quantify the impact of mesophyll dorsiventral asymmetry on the LOP in different species within a growing season.**
  - 4) To describe the seasonal course and variability in leaf biophysical and optical properties related to simultaneous occurrence of leaves in different developmental stage.**

### 3 Summary of published papers

#### 3.1 The effect of leaf stacking on leaf reflectance and vegetation indices measured by contact probe during the season

Eva Neuwirthová, Zuzana Lhotáková and Jana Albrechtová

Sensors 2017, 17, 1202. (IF<sub>2017</sub>: 2.475)

<https://doi.org/10.3390/s17061202>

This paper focuses on the simulation of upscaling from leaf to canopy level using two specific measuring setups – either a single leaf or a stack of leaves – for two broadleaved deciduous tree species: *Populus tremula* and *Salix caprea*. Measurements were taken six times during the 2014 May-October growing season on non-reclaimed post mining sites in the Sokolov region of the Czech Republic. In addition to LOP measurements in two setups, we also assessed leaf biophysical traits: Chl and Car content, equivalent water thickness (EWT) and specific leaf area (SLA) to interpret RS studies.

We observed that leaf stacking increased leaf reflectance in NIR (up to 25%), while it was negligible in VIS and weaker in SWIR than in the NIR. We also found that VIs based on VIS only were not affected by the measurement setup compared to more complex VIs combining wavelengths from VIS and NIR, or VIS and SWIR. Both NIR and SWIR are known to be sensitive to structural traits, and therefore these VIs were affected by measurement setup.

We were also interested in the evolution of the LOP based on different measuring setup during the season. Measuring setup affected VIs that correlated better with chlorophyll content, i.e., these VIs were affected by measuring setup compared to VIs that were not affected by measurement setup.

Our findings that VIs involving wavelengths up to 700 nm, are independent of measuring setup, contribute to findings that improve the interpretation of RS studies. This measurement setup simulates the acquisition of optical properties at the canopy level and simultaneously the concept of increasing LAI used in RTMs. The aim of this methodological study is also to point out that in the case of contact measurement, the number of measured leaf layers matters. From an ecophysiological perspective, we can also confirm that the comparative results in LOP, and leaf biophysical properties of both species indicate the same growing strategy and adaptation to the post mining habitat typical of pioneer tree species, such as *P. tremula* and *S. caprea*.

The data for this study were collected and measured by myself and Zuzana Lhotáková as part of my Master thesis. I performed the sample processing and data analysis, and the text was written by a collective of authors, with myself as the lead author.

### 3.2 Leaf Surface reflectance does not affect biophysical traits modelling from VIS-NIR spectra in plants with sparsely distributed trichomes

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In this study, we chose the model organism *Arabidopsis thaliana* L. and its mutants along with several *Hieracium* species with unique and various surface epidermal structures. Both species notably have very similar internal leaf structures determined by close phylogenetic relationships. However, we were unable to confirm our hypothesis that epidermal traits affect specular reflectance and that leaves with denser and longer trichomes have lower specular and higher total reflectance than the smooth leaves with fewer or no trichomes, nor could we reject this assumption.

Our assumption was based on literature records focusing on noticeable visible variation in leaf structure (internal and surface). However, in our current research, we focused on closely related taxonomic units with the intention of maintaining comparable internal structure that corresponds to a narrow range of surface structures.

In the present paper, we also describe a method for measuring the effect of the surface epidermal structure on specular reflectance in the integrating sphere. We are convinced that the specular reflectance should be further investigated on plant model systems, including glossy leaves and a broader gradient of trichome parameters, but similar internal leaf structure. This study is valuable for refining radiative transfer models using structural traits, such as those incorporating “leaf roughness” as a parameter, e.g., the DLM (Stuckens *et al.*, 2009).

The original idea for this study came from my consulting supervisor, Petr Lukeš, and the hypothesis tested was formulated by a collective of co-authors. The experimental material – seeds of different mutants of *A. thaliana* were provided by my colleagues Petra Cifrová (from the Department of Experimental Plant Biology, Fac. Sci., CUNI) and seeds of *Hieracium* species by Jan Pinc (from the Department of Botany, Fac. Sci., CUNI).

Data for this study were measured by myself and Zuzana Lhotáková, with the help of Miroslav Barták in 2018 and 2019. Biochemical data were processed by me, anatomical analysis was performed by myself and Zuzana Lhotáková, with the help of Drahomíra Bartáková. The leaf surface images (ESEM) was acquired by Jiří Machač (Institute of Botany, Czech Academy of Sciences) and the LOP analysis was performed by myself, Zuzana Lhotáková, and Petr Lukeš. All co-authors contributed to the initial hypotheses and collaborated on the final text, which I coordinated as a lead author.

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### 3.3 Upscaling seasonal phenological course of leaf dorsiventral reflectance in radiative transfer model

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In this study, seasonal leaf development was analysed at the leaf-level of two-sided optical properties, biochemical (Chl, Car contents) and water content, and structural traits (thickness of the entire leaf blade, epidermis: PP, SP, PP/SP ratios) of five common Central European broadleaved tree species (*Betula pendula*, *Fagus sylvatica*, *Acer pseudoplatanus*, *Acer platanoides*, and *Sorbus aucuparia*) with dorsiventral leaf anatomy typical of deciduous trees of temperate forest.

LOP (e.g., reflectance difference between adaxial and abaxial sides, leaf reflectance to transmittance ratio, and fraction of specular component of leaf reflectance) were linked with the laboratory analyses of inner leaf structure during the growing season of 2018. We evaluated the effect of LOP parametrization on canopy BRF by comparing “one-sided” scenarios = reflectance is equal from adaxial (AD) and abaxial (AB) leaf sides and “two-sided” scenarios where LOP are different in AD and AB sides, which is closer to reality observed in nature. The measured LOP were upscaled to canopy level using Discrete Anisotropic Radiative Transfer (DART) model, (Chapter 1.5.4). Neglecting the two-sided optical properties at the leaf level could result in a 20% underestimation of top-of-canopy reflectance.

The measured optical properties vary more over time (due to phenology and leaf development) than among the studied tree species in a specific period. Thus, from a remote sensing perspective, vegetation phenology plays a greater role than individual differences among the species studied. This study can be used to improve the interpretation of RS studies, especially the Chl content at the canopy level. The conclusions of this study indicate that leaf asymmetry should not be neglected when obtaining leaf-level optical properties. This study also highlights that investigating leaf structure through laboratory analyses of their internal structures can improve the interpretation of spectral data at the level of individual canopies or entire forest ecosystems. Incorporating internal leaf structure determined by species specificity, environmental factors, and leaf phenological development during the season into RTMs brings more information about the biological reality observed in-situ into remote sensing studies.

The data for this study were collected during the season 2018 at the Experimental Station Bílý Kříž operated Czech Globe mainly by Petr Lukeš with my assistance (before leaving for the internship in Estonia), Zuzana Lhotáková, Jana Albrechtová and master’s student Zdeňka Češpírová. We - Zuzana, Zdeňka and I, also performed all biochemical and anatomical analyses and helped Petr with the data processing at the leaf level. Petr Lukeš and Růžena Janoutová modelled the collected data in the DART model and transformed our results into the draft story of this paper, where Petr is the lead author. All the co-authors contributed to the manuscript writing.

### 3.4 Leaf age matters in remote sensing: taking ground truth for spectroscopic studies in hemiboreal deciduous trees with continuous leaf formation

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This paper studied the changes in biophysical and optical traits of preformed and neo-formed leaves of three deciduous tree species *Betula pendula*, *Alnus incana* and *Populus tremula* during the growing season and discussed the pooling of interspecific biophysical and spectral data for remote sensing purposes. The main aim was to describe the strong asynchrony of leaf phenological development due to sylleptic growth, which gives rise to neo-formed leaves mainly in the uppermost leaf layer on a canopy. We investigated the effect of this developmental phenomenon occurring in selected tree species on seasonal biophysical and optical leaf properties and relations among them. Our observations confirmed that juvenile leaves, observed on the canopy surface throughout the season, exhibit more stability and higher values of reflectance in VIS compared to mature leaves. The biggest differences in optical properties within the leaf phenological phases (juvenile, mature and senescent leaves) were in the red edge (705 nm) throughout the growing season. Thus, we consider the red edge as an important spectral region in remote sensing studies, not only for stress detection but also for distinguishing leaf phenological phases.

We also present evidence that vegetation indices (VIs) designed to estimate chlorophyll content can show a relationship with PP thickness ( $R^2=0.6$ ), and we observed no effect of growing season on these relations. We consider the most original finding of our study to be the confirmation of the universality and robustness of the relationship between chlorophyll a+b content and palisade parenchyma thickness across leaf phenological stages, species, and sampling day during the growing season. One of the contributions to RS community and to the validation of spectral data is that ground truthing and leaf-level LOP measurements should involve sampling all leaf developmental stages present in the canopy at a given sampling time.

Data for this study were sampled by me and Lea Hallik, my supervisor at the Tartu Observatory, Tartu University, where I stayed for 3-month internship in summer 2018. All sample processing and thorough statistical analysis of the data was done by me using scripts by Andres Kuusk and Joel Kuusk, with the assistance of Lea Hallik and further with Zuzana Lhotáková after my return from Estonia. The text was written by a collective of authors, with me as the lead author.



## 4 Discussion

All the studied tree species included in my thesis are typical European broadleaved trees of temperate and hemiboreal forests and their leaves exhibit a similar dorsiventral structure, i.e., the mesophyll is differentiated into palisade and spongy parenchyma. The tree species studied were: Eurasian aspen (*Populus tremula* L.) and Goat willow (*Salix caprea* L.) in Neuwirthová *et al.* (2017); European beech (*Fagus sylvatica* L.), Silver birch (*Betula pendula* Roth), Norway maple (*Acer platanoides* L.), Sycamore maple (*Acer pseudoplatanus* L.) and Mountain ash (*Sorbus aucuparia* L.) in Lukeš *et al.*, (2020); and Silver birch (*Betula pendula* Roth), Black alder (*Alnus incana* L.), and Eurasian aspen (*Populus tremula* L.) in Neuwirthová *et al.* (2021a);

My last paper (Neuwirthová *et al.*, 2021b), which studied the effect of leaf surface structural traits on LOP, used two evolutionarily closely related groups of the herbaceous plants (*A. thaliana* mutants and *Hieracium* species) with dorsiventral leaf anatomy and comparable leaf internal structure.

In all four studies, two approaches were used to measure LOP with the aim to relate spectral data at the leaf level to their biophysical parameters determined analytically, with particular emphasis on leaf anatomical structure, (terminology in Chapter 1.4.3):

- 1) BRF measurements with a contact probe (CP) Figure 2 in (Neuwirthová *et al.*, 2017), and
- 2) measurements using an integrating sphere: DHRF from adaxial leaf side Figure 6 in (Neuwirthová *et al.*, 2021a), Figure 7 in (Neuwirthová *et al.*, 2021b), DHFR from adaxial and abaxial leaf side Figure 9 in (Lukeš *et al.*, 2020) and DHTF Figure 9 in (Lukeš *et al.*, 2020) from adaxial and abaxial leaf side Figure 10 in the same study at the leaf level.

The studies of broadleaved tree species presented in this thesis contribute to the interpretation of LOP in the context of tree functioning over the season, and therefore required many seasonal field samplings. They all bring more ecophysiological background to RS and RTMs, although each focuses on different details: as I mentioned in Chapter 1.2.3, leaf anatomical structure depends on leaf development, i.e., timing in the growing season and corresponding leaf phenology, an aspect which connects our broadleaved tree studies. Although our study (Neuwirthová *et al.*, 2017) is focused on how vegetation indices are affected by the number of layers in the stack measured for their reflectance, our recent study (Lukeš *et al.*, 2020) discussed the seasonal course of the LOP in dorsiventral leaves and the inclusion of internal leaf heterogeneity in the RTM. The last broadleaved tree study addresses continuous leaf formation during the growing season of sylleptic growing trees (Neuwirthová *et al.*, 2021a).

The most unifying aspect of the above four manuscripts is the study of leaf functional traits - leaf anatomical structure (LT or leaf surface) and photosynthetic pigments - in relation to LOP in the 350-2500 nm region.

### 4.1 Advantages and limitations of the leaf level reflectance acquisition and interpretation

From an ecophysiological point of view, there are some technical and biological limits to the acquisition and interpretation of leaf level optical properties, which will be discussed in this chapter.

The relationships in spectral response associated with leaf biophysical traits in RS studies are influenced by factors such as: canopy structure, proportion of woody elements (Verrelst *et al.*, 2010) or litter and bare soil in the background of vegetation (Malenovský *et al.*, 2008). Compared to

spectroscopic data acquired from field RS studies, laboratory spectroscopy has the advantage that leaf-level reflectance and pigment content are acquired directly from the same leaf sample and then upscaled to the canopy level (Blackburn, 2007). Compared to the study of optical properties at the canopy level, measurements at the leaf level are not subject to any error due to the influence of the measured signal by the state atmosphere and stand structure (among other things), the measured spectral signal is related purely to the structure and composition of the leaves as such.

#### 4.1.1 *LOP are closer to leaf biophysical reality than optical properties from higher hierarchical scales*

A major advantage of laboratory spectroscopy is that relationships of LOP and leaf biophysical traits are usually better derived from the leaf level than from higher hierarchical scales such as the canopy level. In our study (Lukeš *et al.*, 2020), we found a relationship between chlorophyll content and the reflectance on the adaxial side at 550 nm,  $R^2=97$ , which is due to the absence of the above factors. Also, our laboratory spectra at the needle level (Lhotáková *et al.*, 2021) showed a better relation of LOP with biochemically determined Chl and water content compared to remotely acquired spectral data at the canopy level (unpublished data, personal communication Homolová and Švik, 2021, Global Change Research Institute).

#### 4.1.2 *Suitable leaf morphology and leaf size*

The first prerequisite to measure optical properties at the leaf level is the suitable leaf morphology and particularly leaf size, i.e., whether the leaf of interest is large enough to fully cover the FOV of the sensor. Leaf size is a determinant for leaf-level measurement. For example, dwarf woody species with small leaves, such as *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. (Forsstrom *et al.*, 2019), are measured with a goniometer at the canopy level, while species with narrow and small leaves, such as *Juniperus monosperma* [Englem.] Sarg., make it almost impossible to acquire optical properties using a CP or IS at the leaf level (Stimson *et al.*, 2005).

Our selected planar leaves were a morphologically favourable material for LOP measurements compared to coniferous needles, as summarised in methodical study of Einzmann *et al.* (2014). The problem with evergreens arises not only with the spatial arrangement of needles and complex morphology grouped within twigs, but also with material which does not cover the whole FOV, (Yáñez-Rausell *et al.*, 2014). For proper LOP acquisition of needles, it is necessary to correct the measured spectral signal using the so-called "gap fraction" value – the ratio of the coverage of the sample itself in the FOV of the sensor (sampling port of the integration sphere) to the total FOV of the sensor (illumination area of the sampling port of the sphere) (Stimson *et al.*, 2005; Yáñez-Rausell *et al.*, 2014; Olascoaga *et al.*, 2016).

Another technical difficulty when measuring needles using an IS is the detection of negative transmittance values. This phenomena is not unusual when measuring needles by IS, as reported e.g., in the study of Olascoaga *et al.* (2016) or our previous study by Lhotáková *et al.* (2021), as well as by our colleagues from the Global Change Research Institute of Czech Academy of Sciences. Olascoaga *et al.* (2016) pointed out that the measurement of the needle optical properties is prone to error and the measurements depends on gap fractions, morphology of the measured sample, and the cross-sectional shape of individual needles.

#### 4.1.3 *Anisotropy in the leaf venation*

Leaf venation is another structural biological factor which affects the accuracy of leaf spectral data measurements is leaf venation (Grant *et al.*, 1993) and its arrangement either as isotropic or anisotropic. For dorsiventral leaves of dicots exhibiting anisotropic structure, leaf orientation does not play a role in the measurements. Although, mature leaves of grass crop, e.g., corn, possibly cover the sample port size of IS, however, their longitudinal oriented epidermal cells and parallel venation, i.e., structural anisotropy, affect the directionality of reflected light (Comar *et al.*, 2012). This biological aspect can be reduced by measuring the leaf sample with a contact sensor at multiple angles and approximating DHRF from BRF (Comar *et al.*, 2012).

#### 4.1.4 *Heterogeneity in the leaf blade*

In terms of structure and pigment content, the leaf blade is not always homogeneous. Heterogeneity in function, cellular structure, and pigment composition mainly affects photosynthetic function (Takayama *et al.*, 2013). This heterogeneity is explained by several factors, potentially leaf developmental stage or ontogenetic phase corresponding to leaf phenology (e.g., barley leaf develops from the apical towards the basal region (Shaaf *et al.*, 2019)), leaf senescence (Junker and Ensminger, 2016) or the natural appearance of leaf colour determined not only by photosynthetic pigments but also by photoprotective compounds such as anthocyanins (Menzies *et al.*, 2016) or the natural colour patchiness of a leaf blade characterising variegated leaves; displaying light- and dark-green variegation, which can also affect photosynthesis (Konoplyova *et al.*, 2008). To encompass the entire leaf area and capture leaf heterogeneity, it is better to average measurements from more than one location on the leaf blade and avoid the leaf midrib, which is more feasible with a CP (Cavender-Bares *et al.*, 2016; Neuwirthová *et al.*, 2017) than with an IS because the FOV position is more controllable with a CP than an IS.

#### 4.1.5 *Avoiding leaf biophysical changes during the contact measurement*

Thin, delicate leaves, such as those of *A. thaliana*, challenge the use of spectral methods based on recording reflectance or transmittance of a very strong light source because of the possible destruction of chlorophyll and loss of leaf water causing leaf wilting. Overheating caused by a strong lamp in the CP can be prevented by reducing the number of averaging during the measurement period. To the best of our knowledge, CP has not yet been applied to chamber cultivated *A. thaliana* leaves because of possible excessive light damage and leaf overheating in the whole spectrum 300-2500 nm and those spectral data has not been related with chlorophyll content, which has been detected by the biochemical way. The results of our unpublished study show that the strength of the relationship between chlorophyll content detected by biochemical analysis and laboratory spectroscopy using the NDchl index was  $R^2 = 0.57$  for *A. thaliana* wild type and was therefore used as a model to estimate true chlorophyll content based on optical properties. Despite the strong light source of the CP and delicate and thin structure of *A. thaliana* leaves, we achieved non-destructive detection of chlorophyll content in various *A. thaliana* mutants. In the case of delicate leaves, it is better to use non-contact hyperspectral imaging systems similar to those described in the study of Matsuda *et al.* (2012).

## 4.2 Relation of spectrally detected leaf anatomical traits to ecosystem functions

Plant functional traits are commonly used to predict the response of ecosystem functions and provide new insights into global climate change at the ecosystem level (Liu *et al.*, 2019). Functional traits using structural parameters, such as SLA, LT, SP ratio – successfully modelled from LOP in our study (Neuwirthová *et al.*, 2021a), or stomatal size and density belonging to structural traits, respond to leaf strategy of resource capturing, gas exchange and other physiological processes. These structural and morphological functional traits are explained as “economic or hydraulic traits” and can help to understand plant adaptation to changing environments (Liu *et al.*, 2019).

Studies investigating the effect of intercellular space on LOP are not common. For example, Woolley (1971) observed that a larger volume of intercellular space in thicker leaves causes higher reflectance and lower transmittance. Although our studies do not address the effect of intercellular spaces on their LOP, we also measured transmittance in two of them. In (Lukeš *et al.*, 2020) we can use the DLM model to argue for the confirmation of the conclusion of Woolley (1971) that the reflectance-transmittance ratio (R/T) is mainly controlled by the fraction of airspace that increases the R/T ratio at all wavelengths.

It has been suggested that functional traits based on leaf structure are related to ecosystem functions. For example, the study of He *et al.* (2018) investigated SP/LT and PP/SP ratios and found that both anatomical traits are closely related to water use efficiency and gross primary production at the regional scale.

LMA and leaf mass density are closely related to LT (Villar *et al.*, 2013). Thicker leaves usually have higher Chl content (Cao, 2000) and this relation have contribution to Chl content detection (Serrano, 2008), which is consistent with our results (Figure 14 in (Neuwirthová *et al.*, 2021a)). Solar irradiation – its intensity and spectral composition – is one of main factors affecting LMA in shade intolerant plants, as we used in our studies. Due to the gradient in irradiance, the thickness of LMA and PP increases with canopy height (Zhang *et al.*, 2019).

As previously discussed, these ecosystem functions can be investigated using RS studies (Chapter 1.3), and ecosystem functions can be modelled using RTMs based on biophysical inputs at the leaf level (Chapter 1.5.4).

## 4.3 Environmental drivers of the leaf tissue thickness and their implications for LOP

The global-scale meta study on trees and shrubs showed that there are relationships between leaf structure and long-term climatic variables (Niinemets, 2001). For example, LT increases in climates with higher temperature and incident daily solar radiation and temperature, whereas precipitation has no effect on LT. With the prospect of water scarcity and temperature increases due to ongoing climate change, species with thicker and denser leaves may become dominant in many regions in the future (Niinemets, 2001).

LT is determined by various environmental factors such as irradiance during leaf development (Evans *et al.*, 1994), water and mineral nutrient availability, and hydraulic conductance (Aasamaa *et al.*, 2005). LT could also be a marker of plant shade and drought tolerance – thicker leaves usually exhibit better tolerance to drought and high irradiance (Hallik *et al.*, 2019). Thicker leaves with greater surface area of chloroplasts exposed to the intercellular airspaces usually have

better leaf CO<sub>2</sub> transfer conductance and thus higher photosynthetic rates (Evans *et al.*, 1994; Hanba *et al.*, 2001).

As we mentioned in Chapter 1.2.3, the internal structure of leaves is thought to affect their NIR reflectance (Slaton *et al.*, 2001). LT does not only depend on the incident radiation but is also strongly dependent on the genetic basis. Pauli *et al.* (2017) confirmed differences in LT between two genotypes of the Pima Cotton populations grown under the same conditions, which were very similar in leaf structure. Thus, differences in LT across species can be expected. Although other studies have used a wide range of the different plant families and found differences in LT, they did not confirm the relation of LT and leaf reflectance in 800 nm (NIR) (Gausman *et al.*, 1972; Slaton *et al.*, 2001; Sims and Gamon, 2002).

In contrast to the studies just mentioned, which did not confirm strong relation between LT and NIR leaf reflectance, the study by Knapp and Carter (1998) did. The discrepancy in conclusions may be due to the range of samples selected. Slaton *et al.* (2001) measured 48 different alpine angiosperm herbs, and similar structural adaptation to light conditions can be expected in the high altitude region (Trošt Sedej *et al.*, 2020). Gausman *et al.* (1972) investigated the relationship between LT and leaf reflectance at 800 nm in 20 crops separately for each species. Thus, the variability in LT was relatively low. Finally, Sims and Gamon (2002) did not reach any conclusion on the relationship between LT and leaf reflectance at 800 nm, although their study included multiple species with variable leaf structure. In contrast to the above mentioned studies, Knapp and Carter (1998) used 26 species of annual herbs with wide range of growth forms and habitats and concluded that LT is the best predictor for NIR leaf reflectance.

LT is governed by irradiance and mesophyll fraction, intercellular airspace, and spatial distribution of Chl. These are well conserved within plant families and growth habits (Borsuk and Brodersen, 2019), as we observed in juvenile and mature leaves in our study (Neuwirthová *et al.*, 2021a). Even though we also had anatomically comparable material in our studies, we observed visible variability within the internal structure among the species and increasing ratio of the PP/SP driven by the season and leaf phenology (Lukeš *et al.*, 2020). A relatively strong linear dependence between LT and reflectance to transmittance ratio (R/T) at 850 nm was observed in our study (Lukeš *et al.*, 2020) -  $R^2 = 0.6$  - and an inverse dependence of LT on VI MCARI2 we observed in the other study (Neuwirthová *et al.*, 2021a) -  $R^2 = 0.49$ . In the case of the above mentioned study (Neuwirthová *et al.*, 2021a), this dependence was driven by differences in LT and LOP between juvenile and adult leaves. In plants that differ significantly in their leaf biophysical parameters (pigment content and structural traits), differences in LOP are also expected, for example Falcioni *et al.* (2020) observed a strong correlation between absorbance at 1440 nm and LT, although in 1440 nm is one of the absorption maxima of water.

These findings suggest that LT - leaf tissue thickness - is not the only leaf trait affecting NIR leaf reflectance. Environmental drivers (irradiance, CO<sub>2</sub> concentration, temperature, water availability and air humidity) primarily determine leaf biophysical trait development, and its main function - leaf photosynthetic capacity.

#### 4.3.1 Retrieval of photosynthetic capacity based on palisade parenchyma thickness

Kenzo *et al.* (2004) showed a strong positive correlation ( $R^2 = 0.76$ ) between PP thickness and photosynthetic capacity. Photosynthetic capacity is “the maximum rate of carboxylation and

the maximum rate of electron transport” as described by Croft *et al.* (2017). Based on the strong positive relation between photosynthetic capacity and Chl content ( $R^2 = 0.76$ ) Croft *et al.* (2017) produced spatially continuous, seasonal maps of photosynthetic capacity. These maps were created for temperate forest from Landsat satellite data for improved ecosystem modelling.

We have confirmed the relationship between PP thickness and Chl content (see Figure 14 in Neuwirthová *et al.* (2021a)) and therefore it can be assumed that the relationship between PP thickness, chlorophyll content and photosynthetic capacity is also valid for our studied species (Neuwirthová *et al.*, 2021a). Based on this assumption, photosynthetic capacity of our studied species could be retrieved from leaf optical properties or vegetation indices related to chlorophyll content and PP thickness such as Vogelmann or Datt2 vegetation indices. In my opinion, however, there are insufficient data to make decisive conclusion on retrieval of photosynthetic capacity based on PP thickness. For example, Croft *et al.* (2017) also acknowledge that the retrieval of the photosynthetic capacity from RS data have been little investigated.

In our study (Neuwirthová *et al.*, 2021a) we assume that the relationship between LOP and anatomical structure is determined through photosynthetic pigments because we found a relationship between Chl content and LT ( $R^2 = 0.43$ ). However, Falcioni *et al.* (2017) argue that the efficiency of absorption in the green region is determined by the length of the optical pathway and thus that thicker leaves absorb more efficiently in the green region than thin leaves. This is confirmed by the DLM sensitivity analysis in our study (Lukeš *et al.*, 2020), which shows that the R/T ratio in the VIS is mainly controlled by the Chl accumulation in the PP. By using leaf structural parameters (bidirectional leaf side reflectance or LT), vegetation indices can be adjusted and thus significantly improve Chl estimation in a particular plant group, as demonstrated in other studies (Lu *et al.*, 2015 and Zhao *et al.*, 2019) using leaf structural properties to achieve significantly stronger correlation of VIs with Chl content ( $R^2 = 0.92$  and  $R^2 = 0.87$ , respectively). However, this approach only extends the portfolio of the VIs, which is currently quite large (see website Index Database, Chapter 1.5.1) and ultimately should be tested for its universality in other species.

The amount of Chl content, together with the anatomical structure and thickness of individual leaf tissues, is determined by environmental factors: irradiance, temperature, and water availability. All these factors in turn influence the photosynthetic capacity of the leaf. Determining LT and internal anatomical structure is crucial for RTMs and spectral studies, as the results indicate, that: “Leaf anatomy can affect optical patterns regardless of pigment content.” (Falcioni *et al.*, 2020).

#### **4.4 The spatial distribution of the leaf internal tissues affects LOP**

Not only leaf thickness, but also the arrangement and type of internal leaf tissues influence how light pass through and CO<sub>2</sub> diffuse within the leaf and how these processes correspond to light use efficiency (Vogelmann, *et al.*, 1996). How much light is absorbed by the leaf then determines how much carbon can be fixed and assimilated under non-carbon limited conditions (Vogelmann and Gorton, 2014).

##### *4.4.1 Relation of leaf internal structure to light capture*

Epidermal cells commonly focus light (Vogelmann, *et al.*, 1996) and palisade cells also focus light: the columnar shape and arrangement of the palisade cells and chloroplasts inside of the cells

affect light capturing (Kenzo *et al.*, 2004) and minimize light scattering within the leaves, as was well-described in the study of Xiao *et al.* (2016). The aforementioned cell architecture enables light to penetrate deeper into the leaf where more chloroplasts are concentrated (Paradiso and Marcelis, 2012), and intercellular air spaces scatter light and increase the likelihood of light absorption during photosynthesis (Vogelmann and Gorton, 2014).

Leaf mesophyll architecture determines chlorophyll distribution on the leaf cross section. Borsuk and Brodersen (2019) described the observation of a relative chlorophyll increase with increasing leaf depth. Compact leaves with more cell layers reflect more between 600-700 nm (Falcioni *et al.*, 2020). High SP density also increases absorption in the green part of VIS around 550 nm region (Falcioni *et al.*, 2017).

#### 4.4.2 *Relation of leaf internal structure to carbon assimilation*

Leaf porosity and density are related to CO<sub>2</sub> assimilation. In a meta-study at the global level (Niinemets, 2001), it is described that higher leaf density reduces photosynthetic potential due to lower CO<sub>2</sub> conductance. The amount of internal leaf surface area available for CO<sub>2</sub> diffusion, usually expressed as the area of mesophyll cells exposed to intercellular spaces per unit leaf area (Ames/A), is critical for mesophyll CO<sub>2</sub> conductance and, thus, CO<sub>2</sub> assimilation (Slaton and Smith, 2002; Hanba *et al.*, 1999).

Determining Ames/A is not easy, so its correlation with other structural parameters is important: including LT, the amount of intercellular air space, and the size, shape, and density of mesophyll cells (Slaton and Smith, 2002). Not only leaf density expressed as LMA, but also the distribution of chloroplasts in leaves determines the gradient of irradiation and CO<sub>2</sub> within the leaf (Borsuk and Brodersen, 2019). If a greater number of PP cells implies denser leaves, then leaves with thicker PP, and LT, would have lower CO<sub>2</sub> conductance, as confirmed by Evans *et al.* (1994). Leaf expansion is typically associated with increasing PP thickness and Chl accumulation (Neuwirthová *et al.*, 2021a), increasing leaf density and Ames/A, compared to net and CO<sub>2</sub> assimilation rates, which decrease with leaf age (Tosens *et al.*, 2012).

Leaf internal structure also influences photosynthetic rate. Niinemets (2001) observed that a greater LT corresponds to an increased photosynthetic rate per unit area, which is consistent with another observation (Kenzo *et al.*, 2004, mentioned above in Chapter 4.3). This study also showed a strong relation of photosynthetic rate with Ames/A and a weaker, but still significant, positive correlation between photosynthetic rate and LMA. In our studies on deciduous trees growing naturally under similar conditions (Neuwirthová *et al.*, 2017, 2021a) we did not expect a significant difference in porosity of the mesophyll because we did not show differences in LMA values between juvenile and mature leaves.

Leaf anatomical properties also contribute to the refinement of the RTMs, e.g., QSPEC (Ma *et al.*, 2007) and DLM (Stuckens *et al.*, 2009), see Chapter 1.5.4. Based on leaf biophysical traits, growth and transport properties, a virtual leaf can be constructed (Vogelmann, *et al.*, 1996; Retta *et al.*, 2020) as a tool to improve the estimation of difficult-to-measure leaf biophysical traits (photosynthesis and gas exchange) or to upscale these estimates to the ecosystem level.

#### 4.4.3 Effect of leaf dorsiventral asymmetry on the leaf optical properties

As I mentioned in Chapter 1.5.4, LOP of the plants are affected by leaf dorsiventral asymmetry (Vergara-Díaz *et al.*, 2018; Wang, 2020). In our study Lukeš *et al.* (2020) we simulated dorsiventral LOP using leaf-level RT model DLM and performed its sensitivity analysis. We found that: 1) leaf airspace is the main driver of R/T ratio, which is in accordance with the assumption of Woolley (1971), and 2) that ratio of adaxial/abaxial R is driven mainly by the pigments' absorption in VIS. Although we would expect an effect of leaf dorsiventrality on its optical properties only in leaves with palisade and spongy parenchyma differentiation (Wang, 2020; Lukeš *et al.*, 2020), the study of Vergara-Díaz *et al.* (2018) reported different reflectance from the adaxial and abaxial leaf sides also in wheat with dorsiventral leaves with undifferentiated mesophyll. Although they observed that the effect of wheat leaf structure on the reflectance was greater than the effect of irrigation treatment, the differences in dorsiventral spectra were smaller in plants exposed to water stress (Vergara-Díaz *et al.*, 2018).

In our study, we observed an increase of the reflectance signal determined by increasing LAI in canopy simulations (Neuwirthová *et al.*, 2017). According to our findings, it is desirable to upscale two-sided LOP from the leaf level to the canopy level (Lukeš *et al.*, 2020).

Another finding in the study by Van Wittenberghe *et al.* (2015) was that the Chl fluorescence signal, which is much weaker than the reflectance signal, (only 2-5 % of the reflectance signal), was affected by leaf architecture. The partitioning between upward and downward sun-induced chlorophyll fluorescence emission is similar for both equifacial and bifacial leaves: and up to 40 % of the total fluorescence is emitted by the abaxial leaf side. However, in bifacial leaves with more airspaces, downward emitted far-red fluorescence is favoured, compared to a compact equifacial leaves (Van Wittenberghe *et al.*, 2015). Similarly, dorsiventral asymmetry in photosynthesis has been found in *Nicotiana tabacum* L. (Wang, 2020). The authors demonstrated that the photosynthetic apparatus of the adaxial leaf side was more efficient than that of the abaxial side regardless of the light intensity and direct or diffuse light (Wang, 2020).

Not only the dorsiventral character of the leaves, but also solar tracking leaf movement (heliotropism) of the plant leaves affects the assessment of vegetation health conditions by using NDVI (Chávez *et al.*, 2014). Paraheliotropic species from arid regions use leaf movement as a protection against water loss – at midday, leaves face parallel to the incident light. In contrast, hemiboreal and temperate deciduous trees tend to expose their leaves in positions to effectively capture light during the day. Although we did not study paraheliotropic species, as Chávez *et al.* (2014) did, colleagues in our collaborative study modelled the effect of different leaf angle distribution of dorsiventral leaves of temperate trees in DART RTM (Lukeš *et al.*, 2020). Simulations on four tree species with different two-sided optical properties in DART were used with the same structural parametrisation of the canopy to demonstrate the difference between of one- and two-sided scenarios that has been found in the NIR at the peak season, target time and spectral range of many RS studies (Lukeš *et al.*, 2020).

### 4.5 Environmental drivers of the leaf surface structure and their implications for LOP

The leaf surface structure – the epidermis – responds to environmental conditions. The geometrical and biochemical properties of the epidermis and cuticle on peridermal cell walls of epidermal pavement cells, stomata, and trichomes are important for their ecophysiological



functions, such as barriers against water loss preventing cuticular transpiration and pathogen infection. Moreover, the epidermal and cuticular structure influence leaf optical properties (Buda *et al.*, 2009). Cuticular thickness increases during the leaf ontogenetic development (Richardson *et al.*, 2021). Although epidermal cells and cuticle do not contain photosynthetic pigments, optically active compounds such as phenolic compounds may be present, for example in glandular hairs (Gausman and Cardenas, 1969). Trichomes on the epidermis also influence the specular (mirror-like) reflection of the leaf (Grant *et al.*, 1993), which we have investigated in (Neuwirthová *et al.*, 2021b). In our last paper (Neuwirthová *et al.*, 2021b) we tested how structural properties of the epidermal surface - particularly trichomes - affect LOP and how exclusion of specular reflectance improves the relations between leaf spectra and mesophyll pigments. Also Sims and Gamon (2002) concluded that LOP-based estimation of Chl content reached better results with the correction for leaf specular reflectance.

As I mentioned in Chapter 4.4.1: epidermal cells can focus light deeper into the leaf, mainly due to their lens shape (Xiao *et al.*, 2016), and a study by Lin and Ehleringer (1983) reported that the upper epidermis increases leaf reflectance in the 400-700 nm region compared to the leaf after epidermis removal. However, such studies removing the epidermis may be misleading due to artifacts arising from epidermis removal. Special bubble-shaped cells were described by Klančnik *et al.* (2014) as a mechanism for buoyancy leaves in amphibious plants growing in a water column. The volume of epidermis and mesophyll cells negatively correlated with leaf hydraulic conductance (Aasamaa *et al.*, 2005). We did not observe a significant effect of epidermal thickness on reflectance probably because we always measured leaves with one cell layer of epidermis. Changes in epidermis thickness may also contribute to changes in LT response to environmental factors. For example, at high altitudes, epidermal thickness may increase, particularly due to high UV-B radiation (Trošt Sedej *et al.*, 2020)

#### 4.5.1 Leaf cuticle

Lower relative humidity and lower temperature or irradiation induce larger deposits of wax within cuticle (Baker, 1974). Increased cuticular thickness is a xeromorphic adaptation of leaves (Paoletti *et al.*, 2006). Based on the observation of blue wax needles reflecting in ultraviolet and blue wavelengths, a study by Clark and Lister (1975) concluded that reflective scattering caused by epicuticular waxes protects alpine plants from high irradiance, low humidity, and water scarcity. On the other hand, in smooth, hairless leaves, less than 10% of the incident light was reflected from the cuticle (McClendon, 1984). However, my results suggest that cuticle thickness and cuticular wax roughness cannot yet be properly related to leaf reflectance. Considerable difference between shiny leaves of *Prunus laurocerasus* and *Corylus avellana* have been distinguished based on maximum BRDF from several angles (Bousquet *et al.*, 2005) as we also discussed in (Neuwirthová *et al.*, 2021b). There is also a potential to quantify cuticle components in longer IR region (middle IR= 3000-50000  $\mu\text{m}$ ) as recently described by Richardson *et al.* (2021). We conducted a pilot study on leaf reflectance in TIR 2000-18000 nm measured by golden integrating sphere owned by our colleagues from the Czech Globe (Chapter 1.2.5), however, the relations to cuticle thickness have not yet been evaluated.

#### 4.5.2 Leaf trichomes

Similarly to thicker leaf epidermis and cuticle, leaf trichomes (indumentum) are understood as xeromorphic leaf traits that help plants protect themselves from abiotic (high irradiance and low humidity) as well as biotic (herbivorous) factors (Bieras and Sajo, 2009). Early studies have shown a positive correlation of plant trichome density in arid environments with high irradiance (Johnson, 1975), usually associated with UV radiation damage of leaves (Karabourniotis *et al.*, 1993). Several studies (Shull, 1929; Billings and Morris, 1951; Holmes and Keiller, 2002) have described that hairy leaves reflect more light in VIS than leaves without trichomes. We did not confirm this observation in *A. thaliana* because the reflectance of the *glabra* mutant (hairless lineage) was not significantly lower than the reflectance of hairy lineages (Neuwirthová *et al.*, 2021b). We explain this discrepancy by the fact that the studies cited above focused on species from taxonomically very different families with greater variability in indumentum, with the assumption of difference within other biophysical parameters. To avoid difference within biophysical parameters, we selected closely related taxonomic units (*Hieracium* species and *A. thaliana* mutants), which probably caused too narrow range of surface traits. Moreover, *A. thaliana* has a very low trichome density in the wild type (Neuwirthová *et al.*, 2021b).

#### 4.6 Influence of the development of leaf biophysical properties on leaf optical properties

During leaf development, changes in leaf biophysical parameters occur, which also affect the LOP (Neuwirthová *et al.*, 2021a). Leaf development is also associated with changes in biophysical parameters that can be well modelled by LOP and correspond to the characterization of carbon, water and energy fluxes (Yang *et al.*, 2016). Differentiation of leaf function and morphology in developing leaves is affected by environmental factors (e.g., drought stress in arid conditions). Photosynthetic activity and stress resistance have been found to increase during leaf development and a maximum was observed in mature leaves (Liu *et al.*, 2015). A study of Kikuzawa (1995) investigated the seasonal variability of LOP in connection with leaf position on the twig. The authors observed that the rate of leaf development on the twig was determined by irradiance, which also determines canopy architecture. Leaves in exposed canopy layers are better adapted to strong light than leaves in lower canopy layers (Niinemets *et al.*, 2015). They may also differ in LT depending on irradiation – sunlit leaves are known to be thicker and shady leaves thinner. Variation in leaf traits and LOP among the canopy layers due to light availability have also been confirmed (Deepak *et al.*, 2019), who also pointed out the importance of this finding for studies conducted in less dense forests where reflectance is affected by understory. Kikuzawa (1995) observed that when old leaves, located on the older part of a shoot, begin to reduce the rate of photosynthesis, new leaves begin to appear at the top of the shoot, thus vertical elongation of the shoot. Thus, the spatial structure of the canopy could significantly affect the variation in leaf functional traits.

Changes in LOP are closely related to the growing season, which reflects the phenological stages of leaf (Castro and Sanchez-Azofeifa, 2008; Dillen *et al.*, 2012; Yang *et al.*, 2016; Lukeš *et al.*, 2020; Neuwirthová *et al.*, 2021a). Seasonal changes in leaf physiology were taken into consideration when simulating LOP in the PROSPECT model, with the N number-corresponding to mesophyll thickness was increasing during the leaf development and senescence (Demarez, 1999). The authors further point out that the estimation of Chl content in sunlit leaves leads to higher values than in

shaded leaves (Demarez, 1999). The importance of light environment within the canopy during the seasonal changes has also been recently studied (Noda *et al.*, 2021), and importance of these observations have been confirmed for RS studies of the deciduous broadleaved forest.

The phenology of leaf biophysical traits could also be affected by urban heat islands, corresponding to increased ambient temperatures. Notably, the elevated temperature and CO<sub>2</sub> levels due to urban heat islands brings an earlier start of the growing season and a delay in leaf senescence (Wang *et al.*, 2019). During the leaf senescence, photosynthetic pigment content decreases, which is enhanced with the increasing level of the soil sealing (covering the ground by an impermeable material) in urban sites (Wang *et al.*, 2019). Leaf physiological plasticity results in the capacity of vegetation, especially trees, to adapt to urban environment, and this adaptation could be detected by the spectral signal of vegetation (Chi *et al.*, 2022). Spectral monitoring of urban trees helps to improve sustainability of urban vegetation under ongoing climate change (Chi *et al.*, 2022). The influence of urban island conditions on vegetation has become an important topic in tree eco-physiological research. Moreover, a good knowledge of leaf phenology helps us to better understand carbon exchange between land and atmosphere (Wang *et al.*, 2019). Shifts in phenological phases can affect climate through the photosynthesis and carbon sequestration (Peñuelas *et al.*, 2009). Nezval *et al.* (2020) investigated species-specific responses of phenophases to temperature extremes and observed an extension of the growing season due to higher-than-average temperatures within the years 2014-2017.

Similarly to our study (Neuwirthová *et al.*, 2021a), Noda *et al.* (2021) confirmed that understanding biophysical changes within leaf phenology is crucial for improving seasonal canopy reflectance simulations and retrieval of biophysical leaf traits based on them. Proper investigations of leaf phenology reflectance could yield more accurate modelling and interpretations of RS data of plant species with diverse function and structural traits (Noda *et al.*, 2021).

#### **4.7 Potential of LOP for plant water balance evaluation**

Detection of leaf water content (LWC) (Kokaly *et al.*, 2009), equivalent water thickness (EWT) (Yang *et al.*, 2021) or relative water content (RWC) (Eitel *et al.*, 2006) are among the indirect methods to estimate water balance in vegetation (González-Fernández *et al.*, 2015), which is one of the main objectives of many RS studies. Based on the strong water absorption in various parts of NIR region, it is possible to model EWT or, in general, LWC from the LOP (Li *et al.*, 2021).

We have only marginally addressed this issue in our studies, but modelling of dry to fresh leaf weight ratio in *A. thaliana* and *Hieracium* genus from LOP by PLSR (Neuwirthová *et al.*, 2021b) and estimation of leaf water content based on VIs in woody plants (Neuwirthová *et al.*, 2017, 2021a) can be found. Stimson *et al.* (2005) even suggested the possibility of estimating water potential based on water VIs, which they described for coniferous needles, but where water potential measurement could be hampered by technical difficulties in measuring LOP of needles. Thus, we assume that estimation or modelling water potential in planar leaves based on LOP provides scope for future studies.

Detecting water stress in plants using RS methods is important from the perspective of economic losses (Eitel *et al.*, 2006), particularly for crops requiring high temperatures and long season (such as vine plants). Thus, monitoring water stress is crucial to achieve good yields (Rapaport *et al.*, 2014; González-Fernández *et al.*, 2015).

Water deficit and water stress can be reflected by increasing SLA (Zhou *et al.*, 2020). Liu (2003) observed that net photosynthetic rate is negatively correlated with leaf water potential. In contrast to water potential, SLA could be easily retrieved from leaf spectra (e.g., Lukeš *et al.*, 2013; Neuwirthová *et al.*, 2021b).

## 5 Conclusion

During the course of my Ph.D. research, I fulfilled the objectives set out in this thesis by publishing four papers: Two of my papers are more methodological (Neuwirthová *et al.*, 2017; 2021b) and the two others present different applications of LOP in relation to structural traits (Lukeš *et al.*, 2020; Neuwirthová *et al.*, 2021a)

In my first paper (Neuwirthová *et al.*, 2017), I investigated the optical properties of a single or stack of five leaves of deciduous trees as a simulation of the optical properties at the canopy level. In this study, I observed that some commonly used vegetation indices used to detect chlorophyll content are strongly influenced by the structural factor of the stack of leaves, while other vegetation indices are not (Chapter 3.1). In the study Neuwirthová *et al.* (2021b), although we did not observe an effect of the structural properties of the epidermis on LOP in *A. thaliana* mutants and several species of the genus *Hieracium*, especially on the specular component, we were able to model the basic leaf biophysical parameters based on their optical properties using PLSR, which I have not yet found in literature for *A. thaliana* (Chapter 3.2).

Furthermore, my contribution on anatomical analysis in the study Lukeš *et al.* (2020) helped to clarify the effect of dorsiventral leaf asymmetry of deciduous trees on their reflectance from different angles and upscaling this scenario on the top-of-canopy reflectance (Chapter 3.3). In my paper (Neuwirthová *et al.*, 2021a), we observed that not only the part of the season, during which a species is studied, but also its leaf development strategy, is important for interpreting of top-of-canopy reflectance. In the tree species we studied in (Neuwirthová *et al.*, 2021a), we observed young developing leaves to be formed throughout the season, which could affect top-of-canopy reflectance, not just at the beginning of the growing season as would be expected, but throughout the whole growing season (Chapter 3.4).

The present Ph.D. thesis serves primarily as an introduction on remote sensing and laboratory leaf spectroscopy methods (Chapter 1) to my published papers that are part of this thesis and summarized in Chapter 3. In the Scientific background (Chapter 1) I have summarized my knowledge obtained during my Ph.D. studies, primarily to introduce to the broader plant biological community, the issues related to the spectral properties of the leaf in relation to its biophysical properties, processing methods and the evaluation of spectral properties. It was meant as a small guide not only to the papers included in this thesis, but also to other publications focusing on the boundaries between plant biology, ecophysiology, and remote sensing. This thesis also serves as a summary of knowledge on the influence of biochemical, biophysical, and anatomical leaf traits on leaf optical properties at the leaf level that I have gathered during my Ph.D. studies, not only from available literature on remote sensing, but also from my closest collaborators biologists, and from colleagues, remote sensing specialists - some of them my supervisors.

At the same time, I have tried to lead the discussion (Chapter 4) in a more biological direction, focusing not on a discussion of the results of individual papers, which I believe are thoroughly discussed in the individual publications, but on biological interpretations and perspectives that give an ecophysiological dimension to spectroscopic studies and contribute to a better integration of plant physiology, anatomy, spectroscopy, and applied remote sensing.

I firmly hope that my thesis will continue to serve as a summary of knowledge or as a source of literature for all those working at the interface of the fields of plant ecophysiology and remote sensing.

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## 8 Supplement

Supplements are not part of the electronically submitted version of the thesis, because the format in which they were published by individual journals does not allow their conversion to PDF/A. All four publications are attached to the electronic version in the form of an attachment in ZIP format.

List of the Supplements:

- 1) Neuwirthová *et al.*, 2017: The effect of leaf stacking on leaf reflectance and vegetation indices measured by contact probe during the season
- 2) Neuwirthová *et al.*, 2021: Leaf Surface reflectance does not affect biophysical traits modelling from VIS-NIR spectra in plants with sparsely distributed trichomes
- 3) Lukeš *et al.*, 2020: Upscaling seasonal phenological course of leaf dorsiventral reflectance in radiative transfer model
- 4) Neuwirthová *et al.*, 2021: Leaf age matters in remote sensing: taking ground truth for spectroscopic studies in hemiboreal deciduous trees with continuous leaf formation