


# Generality of relationships between leaf pigment contents and spectral vegetation indices in Mallorca (Spain)

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Received: 8 March 2016 / Accepted: 2 July 2017 / Published online: 31 July 2017  
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**Abstract** Vegetation indices are calculated from reflectance data of discrete spectral bands. The reflectance signal in the visible spectral range is dominated by the optical properties of photosynthetic pigments in plant leaves. Numerous spectral indices have been proposed for the estimation of leaf pigment

**Electronic supplementary material** The online version of this article (doi:10.1007/s10113-017-1202-9) contains supplementary material, which is available to authorized users.

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contents, but the efficacy of different indices for prediction of pigment content and composition for species-rich communities is unknown. Assessing the ability of different vegetation indices to predict leaf pigment content we identify the most suitable spectral indices from an experimental dataset consisting of field-grown high light exposed leaves of 33 angiosperm species collected in two sites in Mallorca (Spain) with contrasting leaf anatomy and pigment composition. Leaf-level reflectance spectra were recorded over the wavelength range of 400 – 900 nm and contents of chlorophyll *a*, chlorophyll *b*, total carotenoids, and anthocyanins were measured in 33 species from different plant functional types, covering a wide range of leaf structures and pigment content, five-fold to more than 10-fold for different traits. The best spectral region for estimation of leaf total chlorophyll content with least interference from carotenoids and anthocyanins was the beginning of near-infrared plateau well beyond 700 nm. Leaves of parallel-veined monocots and pinnate-veined dicots had different relationships between vegetation indices and pigments. We suggest that the nature and role of “far-red” chlorophylls which absorb light at longer wavelengths than 700 nm constitute a promising target for future remote sensing studies.

**Keywords** Carotenoids · Chlorophyll · Pigment ratio · Near-infrared · Reflectance · Remote sensing · Visible light

## Introduction

The life on Earth primarily relies on sunlight energy captured by plant pigments to drive the process of photosynthesis. The main photosynthetic pigments in plant leaves are chlorophylls *a* and *b*. The main carotenoids in green leaves of terrestrial plants are  $\alpha$ - and  $\beta$ -carotene, lutein, zeaxanthin, violaxanthin,

antheraxanthin, and neoxanthin (Esteban et al. 2015a, b; Nisar et al. 2015). In plant chloroplasts, pigments are organized into pigment-protein complexes. Proteins coordinate the orientation and distance of pigments relative to each other and, thus, play a crucial role determining the absorption and emission spectra of chlorophylls and carotenoids (Fowler et al. 1992; Bassi and Caffarri 2000). Even as small changes as single amino acid substitutions in a pigment-binding protein can have notable effects on spectral properties of pigment-protein complexes (Morosinotto et al. 2003). Presence of multiple absorption forms of pigments due to differences in protein structures of pigment-binding complexes allows leaves to capture light effectively over a wide spectral region and determines the spectral properties of the whole leaf (Wientjes et al. 2012). The spectral properties of individual pigments are well studied in solution where interactions with other pigments and pigment-protein complexes are absent (Lichtenthaler 1987; Porra 2002), but less is known of spectral properties of pigment-binding complexes as they occur and function in intact leaves (Marin et al. 2011). Despite of the difficulties in purification of pigment-protein complexes, significant advancements have been also made in biochemical and spectroscopic characterization of components of PSI and PSII (Caffarri et al. 2009; Wientjes and Croce 2011) and their acclimation to growth-light spectrum (Hogewoning et al. 2012).

Carotenoids have dual role in capturing and dissipating light energy (Havaux 1998; Havaux et al. 1998; Demmig-Adams and Adams 2006). The function of different carotenoids is closely associated with their exact location in the photosynthetic apparatus (Polívka and Sundström 2004). Carotenoids absorb green-blue light and transfer the excitation energy to the chlorophylls. At the same time, they also participate in the dissipation of excess absorbed energy and quenching of chlorophyll excitations (Marin et al. 2011). In shade conditions, the relative share of lutein increases, while at high-light availability, the pools of  $\beta$ -carotene and especially xanthophyll cycle pigments are increased (Niinemets et al. 1998; Krause et al. 2001; Demmig-Adams and Adams 2006; Matsubara et al. 2009; Esteban et al. 2015a, b). Hence, the change in leaf total carotenoid content or carotenoid to chlorophyll ratio can be modest during light acclimation corresponding to multiple contrasting adjustments in pigment composition (Hallik et al. 2012). Changes in chlorophyll *a* to *b* ratio can also reflect multiple adjustments in response to light quality and quantity. Ratio of two photosystems (PSI and PSII) as well as the size of light-harvesting antenna complexes modify chlorophyll *a* to *b* ratio (Hansen et al. 2002; Kitajima and Hogan 2003; Fan et al. 2007; Niinemets 2010a). Apart from changes in incident light availability, there is also important light gradient within the leaf, associated both with changes in carotenoid to chlorophyll ratio and chlorophyll *a* to *b* ratio (Terashima and Hikosaka 1995 for a review). Thus, the whole-leaf average leaf pigment composition can be

importantly driven by leaf internal architecture that smooths the effects of differences in incident light availability on foliage pigments.

The effect of photosynthetic pigments is dominating the reflectance signal in the visible spectral region. Remote sensing methods allow quantifying pigment contents and composition by reflectance measurements at various spatial scales ranging from observations at the single leaf level to spaceborne measurements for assessment at whole ecosystem level (Huete et al. 2002; Ustin et al. 2009). Measurements from satellite platforms provide global coverage and form long time series from continuous revisits of the same pixels. New ESA satellite mission SENTINEL 2 with two satellites (Sentinel-2A and Sentinel-2B) has revisiting frequency of 5 days and 10-m spatial resolution for optical bands. Landsat 8 which is the latest in a continuous series of land remote sensing satellites that began in 1972 offers 16-day repetitive Earth coverage, and its multispectral bands have 30-m spatial resolution. Both satellite programs offer standard data products at no charge. An unprecedented amount of Earth Observation (EO) data is now available for researchers and society. To achieve the wider use of EO data, great effort has been made to provide user-friendly access to satellite data. Copernicus (previously known as Global Monitoring for Environment and Security (GMES)) is a European Union program aimed at developing free and openly accessible information services based on satellite EO and in situ data. USGS is a scientific agency of the US government facilitating free access to EO data products. Vast amount of EO data is currently available for detecting and monitoring changes at both regional and global scales. Frequent revisiting time and high spatial resolution of new optical satellite missions makes the satellite-based remote sensing to the most powerful technology for monitoring vegetation response to environmental change at regional scales. Light absorption in photosynthetic pigments is the main signal which can be traced with optical satellite missions for monitoring the changes in the state of vegetation.

This said, ground validation with in situ measurements is the bottleneck that limits the applications of satellite-level remote sensing for estimation of the biophysical parameters of vegetation. The amount and the quality of ground validation data determine the quality of estimation of the vegetation characteristics from EO data. Top of canopy as well as single leaf level reflectance measurements with leaf-clips or integrating spheres are used for validating the relationships between vegetation traits and EO data. The topic of the current article is the relationship between reflectance signal and vegetation characteristics at single leaf level.

The term “vegetation index” originates from 1960s to 1970s when the first studies exploring the possibilities of using satellite images for vegetation monitoring were conducted and have been in wide use ever since. This term refers to any transformation of two or more discrete spectral bands for

assessing vegetation properties either at leaf or canopy levels. Vast amount of different vegetation indices has been developed to estimate leaf pigment contents (Appendices 1 and 2). The indices have been developed for different types of vegetation, often based on a limited range of foliage structures, and to our knowledge, there has been no comprehensive analyses testing the predictive capacity of different spectral indices using the same foliar dataset that provides an extensive range of leaf structures and pigment contents for mature field-grown, high-light-exposed leaves. Due to correlated variations among leaf photosynthetic pigment pools, accurate estimation of different pigments from leaf spectral data is a formidable task. Our aim was to deal with the diversity of spectral indices and address the controversy that different studies suggest different wavelength regions and prediction formulas for estimating the contents of the same pigments or pigment classes and their ratios.

We compiled an extensive list of existing vegetation indices and prediction formulae for leaf pigment content (152 indices; Appendix 1). We conducted leaf level reflectance and pigment content measurements in leaves of 33 species with contrasting leaf anatomy to generate an independent test dataset of leaf traits for assessment of the universality of different vegetation indices and spectral regions proposed so far for leaf pigment content estimation. As the reflectance signal measured by aerial or space-borne sensors would be dominated by high-light-exposed upper canopy leaves of multiple species, we used only high-light-developed leaves. Our particular emphasis was to separate the individual and combined effects of different pigment classes and ratios (e.g., chlorophyll *a/b* ratio and chlorophyll to carotenoid ratio) on leaf reflectance data.

## Material and methods

### Plant sampling

Foliage of 33 angiosperm species was collected in two sites in Mallorca (Balearic Islands, Spain) in December 2010 (Table 1 for the list of species), the Sóller Botanic Garden (39° 45' 53" N, 2° 42' 75" E; 47 m of elevation), and the garden of the University of Balearic Islands (39° 38' 32" N, 2° 38' 37" E; 95 m of elevation) that allowed for collection of large number of plant species growing together in similar environmental outdoor conditions. In both sites, the climate was typically Mediterranean, characterized by seasonal variation of the photoperiod, high temperatures, and low rainfall during the summer. The average annual precipitation was 460 and 617 mm, the mean maximum temperature of the warmest month (July) was 31.4 and 32.4 °C, and mean minimum temperature of the coldest month (January) was 8.3 and 5.9 °C at the University and in Sóller, respectively. Climatic data were obtained from

weather station 7450 Groweather, situated in the experimental field at the University of Balearic Islands, and a climatic station Sa Vinyassa situated in Sóller.

Only sunlit leaves from fully exposed plants growing outdoors were collected. From each species, three healthy green leaves were sampled. Additionally, red-colored leaves containing higher amounts of anthocyanins were collected from *Lantana camara* L. and *Protea* sp.

### Measurement of leaf optical properties

Leaf reflectance spectra were measured with an integrating sphere AvaSphere-50-REFL (Avantes BV, the Netherlands) and spectrophotometer SpectraVista HR-1024 (Spectra Vista Corporation, USA) using the light source AvaLight-HAL and a white spectral on reference. Spectra were recorded in 350–2500-nm range (FWHM 3.5 nm at the visible region). Reflectance spectra were smoothed using Savitzky-Golai method (Savitzky and Golay 1964), which is based on simple polynomial least squares calculations. Polynomial degree was set 2 and filter size 19.

### Determination of leaf pigment contents and structural characteristics

Two disks with a fixed diameter of 1 cm were cut from each sampled leaf after reflectance measurements. One leaf disk was used for determination of chlorophyll and carotenoid concentrations. The second leaf disk was used for quantification of anthocyanins. Concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids in leaf samples were determined by high-pressure liquid chromatography (HPLC; Agilent 1200) as described in Oprüş et al. (2013) using the method of Niinemets et al. (1998). Leaf disks were homogenized in liquid nitrogen, and pigments were extracted in 100% acetone with added calcium carbonate. Pigments in the extracts were separated in a reverse-phase C18 column (Agilent ZORBAX Eclipse XDB) with gradient elution of acetone and water. Concentration of total carotenoid (Car) was calculated as the sum of  $\beta$ -carotene, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, and lutein. Anthocyanins were quantified by extraction in methanol:HCl:H<sub>2</sub>O mixture (90:1:1 v:v:v) acidified with HCl (1% vol). The extracts were centrifuged and assessed spectrophotometrically (Shimadzu dual-beam spectrophotometer UV2550PC, Shimadzu Corporation, Kyoto, Japan) at 529 nm (anthocyanin absorbance) and 650 nm (chlorophyll absorbance used for correction) (Sims and Gamon 2002).

Thickness of fresh leaf blade was measured with a caliper avoiding the main veins. Fresh leaf images were scanned for area measurements at 300 dpi, and leaves were dried at 80 °C to determine the ratio of leaf dry mass per unit area (LMA).

**Table 1** Description of studied species and the main leaf traits: total chlorophyll content per leaf area (Chl *a* + *b*), chlorophyll *a* to chlorophyll *b* ratio (Chl *a/b*), total carotenoid (Car) and anthocyanin (Anth) content per leaf area, leaf blade thickness (FLT), and leaf dry mass per area (LMA)

No.	Group (class)	Family	Species	Form	Chl <i>a</i> + <i>b</i> (g/m <sup>2</sup> )	Chl <i>a/b</i>	Car (g/m <sup>2</sup> )	Anth (g/m <sup>2</sup> )	FLT (mm × 10 <sup>-2</sup> )	LMA (g/m <sup>2</sup> )
1	Monocots ( <i>Liliopsida</i> )	<i>Arecaceae</i>	<i>Chamaerops humilis</i>	Tree	0.482 ± 0.052	3.44 ± 0.13	0.214 ± 0.038	0.018 ± 0.002	32.67 ± 2.52	175.44 ± 6.92
2		<i>Hyacinthaceae</i>	<i>Urginea maritima</i>	Herb	0.949 ± 0.066	2.98 ± 0.05	0.331 ± 0.026	0.007 ± 0.007	81.67 ± 1.53	98.46 ± 4.26
3		<i>Iridaceae</i>	<i>Iris cretensis</i>	Herb	0.558 ± 0.116	3.26 ± 0.08	0.212 ± 0.061	0.012 ± 0.002	63.33 ± 10.41	95.18 ± 18.83
4		<i>Ruscaceae</i>	<i>Dracaena drago</i>	Tree	1.148 ± 0.103	2.95 ± 0.01	0.392 ± 0.0	0.006 ± 0.004	125.00 ± 13.23	184.73 ± 5.95
5		<i>Steruliaceae</i>	<i>Sterilizita reginae</i>	Herb	1.066 ± 0.134	3.07 ± 0.14	0.369 ± 0.047	0.013 ± 0.004	47.33 ± 2.08	117.55 ± 6.91
6	Dycots ( <i>Magnoliopsida</i> )	<i>Aceraceae</i>	<i>Acer opalus</i> ssp. <i>granatense</i>	Tree	0.307 ± 0.017	2.66 ± 0.08	0.111 ± 0.006	0.029 ± 0.001	19.00 ± 1.00	71.69 ± 4.95
7		<i>Apocynaceae</i>	<i>Neortium oleander</i>	Shrub/tree	0.674 ± 0.012	3.44 ± 0.10	-	0.018 ± 0.001	42.33 ± 2.52	196.80 ± 7.05
8		<i>Aquifoliaceae</i>	<i>Ilex aquifolium</i>	Shrub/tree	0.91 ± 0.142	3.33 ± 0.24	0.318 ± 0.066	0.013 ± 0.001	50.00 ± 3.61	189.53 ± 22.26
9		<i>Araliaceae</i>	<i>Hedera helix</i>	Liana (climbing)	0.945 ± 0.195	3.05 ± 0.04	0.329 ± 0.1	0.016 ± 0.01	43.67 ± 9.02	144.92 ± 46.04
10		<i>Asteraceae</i>	<i>Pilosostemon casabonae</i>	Herb	0.937 ± 0.062	3.23 ± 0.11	0.288 ± 0.023	0.02 ± 0.003	43.00 ± 2.00	130.08 ± 6.71
11		<i>Crassulaceae</i>	<i>Aeonium percarneum</i>	Succulent	0.227 ± 0.105	2.64 ± 0.16	0.207 ± 0.055	0.024 ± 0.005	501.67 ± 2.89	267.38 ± 13.85
12		<i>Ericaceae</i>	<i>Arbutus unedo</i>	Shrub/tree	0.625 ± 0.073	3.40 ± 0.07	0.261 ± 0.013	0.017 ± 0.002	37.67 ± 0.58	157.56 ± 5.32
13		<i>Fabaceae</i>	<i>Ceratonia siliqua</i>	Shrub/tree	0.609 ± 0.027	3.47 ± 0.02	0.259 ± 0.009	0.043 ± 0.004	34.00 ± 2.65	127.37 ± 6.62
14		<i>Fagaceae</i>	<i>Quercus ilex</i>	Tree	0.803 ± 0.05	3.57 ± 0.12	0.317 ± 0.003	0.02 ± 0.002	44.67 ± 1.53	200.00 ± 6.67
15		<i>Lamiaceae</i>	<i>Stachys eretica</i>	Shrub	0.743 ± 0.131	3.04 ± 0.13	0.231 ± 0.026	0.021 ± 0.005	52.00 ± 3.46	70.32 ± 10.70
16		<i>Lamiaceae</i>	<i>Phlomis fruticosa</i>	Shrub	0.663 ± 0.082	3.44 ± 0.20	0.236 ± 0.02	0.016 ± 0.007	57.50 ± 3.54	102.42 ± 5.64
17		<i>Magnoliaceae</i>	<i>Magnolia excelsa</i>	Tree	0.292 ± 0.091	3.03 ± 0.03	0.168 ± 0.031	0.028 ± 0.018	53.33 ± 2.89	201.48 ± 8.36
18		<i>Mahvaceae</i>	<i>Hibiscus rosa-sinensis</i>	Shrub/tree	0.252 ± 0.014	2.39 ± 0.06	0.105 ± 0.011	0.027 ± 0.002	36.67 ± 1.53	67.18 ± 4.77
19		<i>Mahvaceae</i>	<i>Lavatera olbia</i>	Shrub	0.456 ± 0.011	3.40 ± 0.11	0.186 ± 0.008	0.018 ± 0.004	32.00 ± 4.36	81.67 ± 11.82
20		<i>Melastomaceae</i>	<i>Melianthus major</i>	Shrub	0.318 ± 0.045	3.39 ± 0.13	0.129 ± 0.014	0.019 ± 0.003	23.33 ± 0.58	45.76 ± 3.46
21		<i>Myrtaceae</i>	<i>Eucalyptus camaldulensis</i>	Tree	0.244 ± 0.029	3.28 ± 0.16	0.094 ± 0.012	0.022 ± 0.002	45.67 ± 3.79	204.18 ± 16.14
22		<i>Pittosporaceae</i>	<i>Pittosporum tobira</i>	Shrub	0.758 ± 0.069	3.18 ± 0.01	0.285 ± 0.021	0.023 ± 0.01	42.67 ± 2.52	190.79 ± 7.63
23		<i>Plumbaginaceae</i>	<i>Limonium sventenii</i>	Shrub	0.329 ± 0.032	3.33 ± 0.08	0.115 ± 0.009	0.02 ± 0.005	44.33 ± 6.03	58.13 ± 2.58
24		<i>Proteaceae</i>	<i>Protea</i> sp.	Shrub	0.261 ± 0.017	4.14 ± 0.04	0.089 ± 0.007	0.074 ± 0.022	56.67 ± 1.53	216.08 ± 11.29
24a		<i>Proteaceae</i>	<i>Protea</i> sp.	Shrub	0.105 ± 0.047	4.47 ± 0.81	0.036 ± 0.015	0.314 ± 0.117	57.33 ± 1.53	233.61 ± 10.57
25		<i>Ranunculaceae</i>	<i>Ranunculus bullatus</i>	Herb	0.218 ± 0.042	2.28 ± 0.22	0.079 ± 0.013	0.022 ± 0.004	31.00 ± 1.00	42.12 ± 8.43
26		<i>Rhamnaceae</i>	<i>Rhamnus alaternus</i>	Shrub	0.589 ± 0.03	3.29 ± 0.09	0.236 ± 0.01	0.021 ± 0.001	30.33 ± 0.58	133.03 ± 5.61
27		<i>Rosaceae</i>	<i>Eriobotrya japonica</i>	Shrub/tree	0.555 ± 0.108	3.12 ± 0.09	0.231 ± 0.031	0.027 ± 0.007	37.33 ± 0.58	174.92 ± 3.64
28		<i>Rutaceae</i>	<i>Citrus reticulata</i>	Tree	0.636 ± 0.11	2.91 ± 0.14	0.262 ± 0.019	0.014 ± 0.006	41.67 ± 2.89	174.11 ± 8.16
29		<i>Urticaceae</i>	<i>Urtica membranacea</i>	Herb	0.341 ± 0.019	3.15 ± 0.04	0.122 ± 0.012	0.023 ± 0.003	14.00 ± 1.00	31.74 ± 6.11
30		<i>Valerianaceae</i>	<i>Centranthus ruber</i>	Herb	0.462 ± 0.031	3.39 ± 0.07	0.17 ± 0.018	0.019 ± 0.001	41.00 ± 2.65	49.86 ± 3.72
31		<i>Verbenaceae</i>	<i>Lantana camara</i>	Shrub	0.308 ± 0.018	3.20 ± 0.09	0.166 ± 0.006	0.03 ± 0.01	30.00 ± 0	74.71 ± 10.05
31a		<i>Verbenaceae</i>	<i>Lantana camara</i>	Shrub	0.344 ± 0.006	3.39 ± 0.04	0.185 ± 0.004	0.082 ± 0.033	43.33 ± 7.64	104.77 ± 11.64

## Review of vegetation spectral indices and assessment of the capacity of different indices to predict foliage pigment contents

To validate the performance of existing vegetation indices (VIs) in estimating leaf pigment contents, we selected 152 published VIs that can be calculated from the reflectance or from the first derivative of reflectance at one or more fixed wavelengths in 400–1000-nm spectral region (i.e., do not require integration of spectra and searching maxima). Among 152 VIs selected from literature, 83 were originally developed for predicting Chl  $a + b$ , 18 for Chl  $a$ , 13 for Chl  $b$ , 21 for Car, 2 for Chl  $a/b$  ratio, and 15 for Car/Chl or Car/Chl  $a$  ratio (Appendices 1 and 2).

To assess the performance of different VI formula across the whole visible spectral region and near-infrared plateau (400–900 nm), we calculated six types of VIs as simple mathematical combinations of reflectance ( $R$ ) or its first derivative ( $D$ ) at two different wavelengths ( $\lambda_1$  and  $\lambda_2$ ): (1) simple difference  $SD = R_{\lambda_1} - R_{\lambda_2}$ , (2) simple ratio  $SR = R_1/R_2$ , (3) normalized simple difference  $NSD = (R_1 - R_2)/(R_1 + R_2)$ , (4) derivative difference  $DD = D_1 - D_2$ , (5) derivative ratio  $DR = D_1/D_2$ , and (6) normalized derivative difference  $NDD = (D_1 - D_2)/(D_1 + D_2)$ . In this analysis, every wavelength combination through 400- to 900-nm spectral region was tested for its capacity to predict leaf pigment contents using correlation analyses.

The main dataset used for assessing the relationships between leaf pigment content and spectral properties consisted only green (excluding visually red) high-light-grown leaves. For calculation of root-mean-square error (RMSE) among estimated and predicted values, the main dataset was divided among “study” and “test” datasets. The study dataset contained 2/3 samples of each species, and 1/3 samples was left for the test dataset. The study dataset was used for developing linear regressions between VI and pigment content or the ratio of pigments. If other prediction equation was suggested by the author of specific VI, it was used. These formulas based on linear regressions or other equations were applied to predict the target variable in the test dataset and to calculate RMSE. To evaluate the sensitivity of different VIs to anthocyanin content, we added leaves with high anthocyanin content (visually red-colored leaves) to the test dataset. If this caused RMSE to increase at least by 10%, the given VI was labeled as sensitive to the presence of elevated levels of anthocyanins (Table 2 and Appendix 1).

### Grouping of species

Studied species can be divided into groups of woody and herbaceous plants based on growth form and stem longevity, grouped as evergreen and deciduous based on leaf longevity or classified among monocots and dicots based on the number

of embryonic leaves that is further characteristically associated with major differences in the structure of adult leaves. Monocot leaves have weakly differentiated mesophyll and parallel or palmate venation, while dicot leaves have characteristic separation of spongy and palisade mesophyll layers and typically have pinnate venation. Major agricultural grass crops are monocots. Among dicots, we separately analyzed leaves with high anthocyanin content and the succulent species *Aeonium percarneum*. The succulent species had notably higher leaf water content and leaf thickness compared to other species, but its LMA and total pigment pools did not differ from overall averages of these traits across species (Table 1). Due to higher carotenoid to chlorophyll ratio (Car/Chl), this species had to be removed in some statistical analysis to avoid mixing the effects of Car/Chl ratio and leaf water content. The removal of the succulent species is specifically indicated in each occasion.

## Results

### General relationships among leaf traits

Leaf total chlorophyll (Chl  $a + b$ ) content ranged from 0.1 to 1.15 g m<sup>-2</sup> among studied species, and the variation in total Car content was about an order of magnitude, from 0.04 to 0.4 g cm<sup>-2</sup> (Table 1). However, chlorophyll  $a$  to  $b$  (2.4–3.4) and total carotenoid to chlorophyll ratio (0.3–0.6; *A. percarneum* 1) varied less than total photosynthetic pigment pool sizes. Concentration of anthocyanins remained below 0.03 g m<sup>-2</sup> in most of green leaf samples, while in red-colored leaves, it reached up to 0.3 g m<sup>-2</sup> (Table 1).

For the majority of species, leaf thickness was within a range of 0.2 to 0.5 mm (Table 1). However, the thickness was about 1 mm in some monocots and even up to 5 mm in the succulent species *A. percarneum* (Table 1). LMA ranged from 32 to 267 g m<sup>-2</sup> and leaf fresh mass per unit area (FLMA) from 159 to 3580 g m<sup>-2</sup>. Thus, extensive variability in all studied leaf traits was observed across the species in this study.

FLMA described 98% of the variability in leaf thickness (Fig. 1), while LMA predicted only 20% of variability in leaf thickness in our dataset. Leaf total pigment pools were strongly correlated with each other. Leaf total chlorophyll content described 87% of variability in leaf total carotenoid content (Fig. 2).

### Efficacy of available VIs for predicting leaf total chlorophyll content

The best-performing VIs were describing more than 80% of variability in total chlorophyll content in green healthy leaves and were not significantly affected by the presence of

**Table 2** Summary of literature-based vegetation indices in Appendices 1 and 2

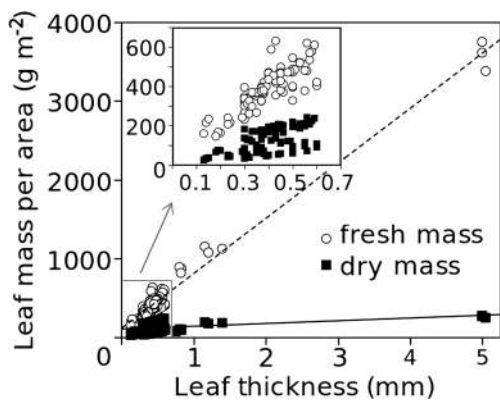
Original target	Name, if so	Authors	Type	Formula	Model	$R^2$	RMSE	Slope diff. <sup>a</sup> (%)	Closest target	Sensitivity
Chl <i>a + b</i>	mNDVI8	Mutanga and Skidmore (2004)	NSD	$(R755 - R730)/(R755 + R730)$	L	0.824	9.857	48	Ca	
	mNDVI1	Mutanga and Skidmore (2004)	NSD	$(R755 - R745)/(R755 + R745)$	L	0.811	10.793	44	Ca	
	NDRE	Barnes et al. (2000)	NSD	$(R790 - R720)/(R790 + R720)$	L	0.809	10.120	51	Ca	
	–	IN: Thenkabail et al. (2013)	NSD	$(R855 - R720)/(R855 + R720)$	L	0.800	10.285	52	Ca	
	ND 780, 712	Féret et al. (2011)	NSD	$(R780 - R712)/(R780 + R712)$	$40.65x^2 + 121.88x - 0.77$	0.792	10.104	50	Ca	
Chl <i>a</i>	–	Imanishi et al. (2010)	SR	R770/R740	L	0.806	8.465	43	Ca	
	–	Imanishi et al. (2010)	ind	$(R760 - R720)/(R725 - R720)$	$50x^2 - 15.4x$	0.801	9.985	29	Ca	
	–	Datt (1999)	ind	$(R850 - R710)/(R850 - R680)$	$\times 2.9564$	0.777	8.447	44	Ca	
	chINDI	Gitelson and Merzlyak (1994)	NSD	$(R750 - R705)/(R750 + R705)$	L	0.776	8.063	46	Ca	
	–	Gitelson and Merzlyak (1994)	SR	R750/R705	L	0.728	8.260	43	Ca	
Chl <i>b</i>	–	Imanishi et al. (2010)	SR	R810/R730	L	0.746	2.681	57	Ca	
	–	Maccioni et al. (2001)	SR	R556/R750	$10 \times$	0.639	3.032	55	Ca	anth
	–	Imanishi et al. (2010)	ind	$(R455 - R745)/R590$	$-0.996x^2 + 20.3x$	0.619	3.358	40	Ca + b	anth
	PSNDb1	Blackburn (1998)	NSD	$(R800 - R635)/(R800 + R635)$	$e^{4.3006x}$	0.578	3.659	41	Ca + b	anth
	–	Imanishi et al. (2010)	NSD	$(R855 - R735)/(R855 + R735)$	$e^{15x}$	0.573	3.614	64	Ca	
Car	ND 800, 530	Féret et al. (2011)	NSD	$(R800 - R530)/(R800 + R530)$	$8.09x^2 + 11.18x - 0.38$	0.640	4.471	30	Ca	anth
	RI 530, 800	Féret et al. (2011)	SR	R530/R800	$24.69x^2 - 39.89x + 17.74$	0.634	4.513	32	Ca	anth
	–	Datt (1998)	ind	$R672/(R550 \times R708)$	$\times 0.7488$	0.480	5.371	na	Ca	anth
	–	Datt (1998)	ind	$R860/(R550 \times R708)$	$\times 1.2396$	0.452	5.189	32	Ca	anth
	–	IN: Thenkabail et al. (2013)	NSD	$(R550 - R687)/(R550 + R687)$	L	0.362	6.072	na	Ca	anth
Chl <i>a/b</i>	–	Nicotra et al. (2003)	SR	R696/R770	L	0.050	0.258	na	Ca	anth
	–	Siebke and Ball (2009)	ind	Ind 626, 603, 647, 552	L	0.024	0.291	na	na	anth
Car/Chl	SIPI1	Peñuelas et al. (1995)	ind	$(R800 - R445)/(R800 - R680)$	$e^{-0.481x}$	0.244	0.208	na	na	
	SIPI1a	Blackburn (1998)	ind	$(R800 - R445)/(R800 - R680)$	$\ln(x)$	0.241	0.209	na	na	
	SIPI3	Blackburn (1998)	ind	$(R800 - R470)/(R800 - R680)$	$\ln(x)$	0.218	0.211	na	na	
	SIPI2	Peñuelas et al. (1995)	ind	$(R800 - R505)/(R800 - R590)$	$e^{-0.263 \times x}$	0.218	0.209	na	Ca	
	SIPI2a	Blackburn (1998)	ind	$(R800 - R505)/(R800 - R590)$	$\ln(x)$	0.214	0.209	na	Ca	

Five best performing vegetation indices from literature originally developed for predicting (1) total chlorophyll content (Chl *a + b*), (2) chlorophyll *a* (Chl *a*), (3) chlorophyll *b* (Chl *b*), (4) total carotenoid content (Car), (5) chlorophyll *a* to chlorophyll *b* ratio (Chl *a/b*), and (6) carotenoids to chlorophyll ratio (Car/Chl)

<sup>a</sup> Difference between monocotyledons and dicotyledons

anthocyanins (Appendix 1). However, a clear pattern emerged that all top-ranking VIs were utilizing wavelengths beyond 700 nm so that both the predictive and the reference wavelengths were located in the near-infrared part of the spectrum (Table 2 and Appendix 1). It also appeared that nearly all VIs

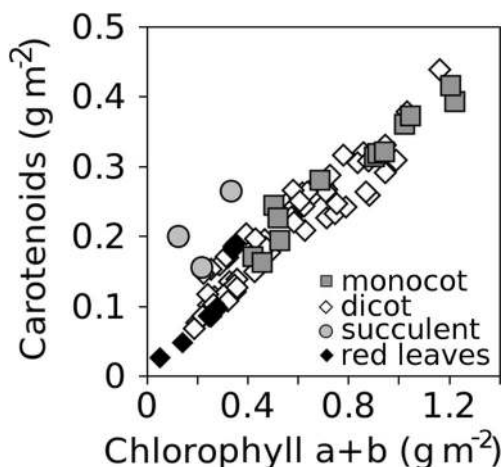
developed originally for predicting total leaf chlorophyll content were slightly more strongly correlated with Chl *a* content than with the sum of Chl *a* and Chl *b*. VIs which used reflectance near-chlorophyll absorption maxima tended to perform the worst in predicting leaf chlorophyll content (Appendix 1).



**Fig. 1** Dependence of leaf fresh mass (FLMA; empty circles) and dry mass per area (LMA; black squares) on leaf thickness. Trend lines depict linear regressions

### Capacity of available VIs for predicting chlorophyll *a* and *b* contents separately and chlorophyll *a* to *b* ratio

The tested VIs originally developed for Chl *a* estimation showed always a stronger relationship with Chl *a* than with total leaf chlorophyll content, suggesting that these indices indeed are specific to Chl *a* (Appendix 1). Although the best indices developed specifically for Chl *a* prediction were able to describe up to 80% of Chl *a* variability (e.g., R770/R740 by Imanishi et al. 2010), few indices originally developed for total chlorophyll *a* + *b* (or total green biomass) estimation were performing even better for Chl *a* describing up to 83% of Chl *a* variability (e.g., (R755 – R730)/(R755 + R730) by Mutanga and Skidmore 2004). Most of VIs originally developed for Chl *b* estimation were more strongly correlated with Chl *a* than with Chl *b* (Appendix 1). Neither of the two VI found in literature for estimation of Chl *a/b* ratio showed any



**Fig. 2** Relationship between leaf total chlorophyll and total carotenoid contents. Separate symbols denote monocotyledons (squares) and dicotyledons (rhombs). The only succulent species in dataset (*Aeonium percarneum*) is marked with circles, and visually red-colored leaves collected extra due to higher anthocyanin content are marked with black rhombs

capacity for prediction of Chl *a/b* ratio in high-light-exposed leaves of our study (Table 2).

### How well available VIs can predict leaf total carotenoid content or chlorophyll to carotenoid ratio

Better-performing indices developed for Car were capable of describing up to 64% of variability in leaf total Car content (e.g., (R800 – R530)/(R800 + R530) by Féret et al. 2011). Despite being developed for Car, these indices were more strongly correlated with leaf chlorophyll content, particularly with Chl *a*. VIs originally developed for assessing carotenoid to chlorophyll ratio (Car/Chl) were relatively weakly correlated with Car/Chl ratio in our dataset.

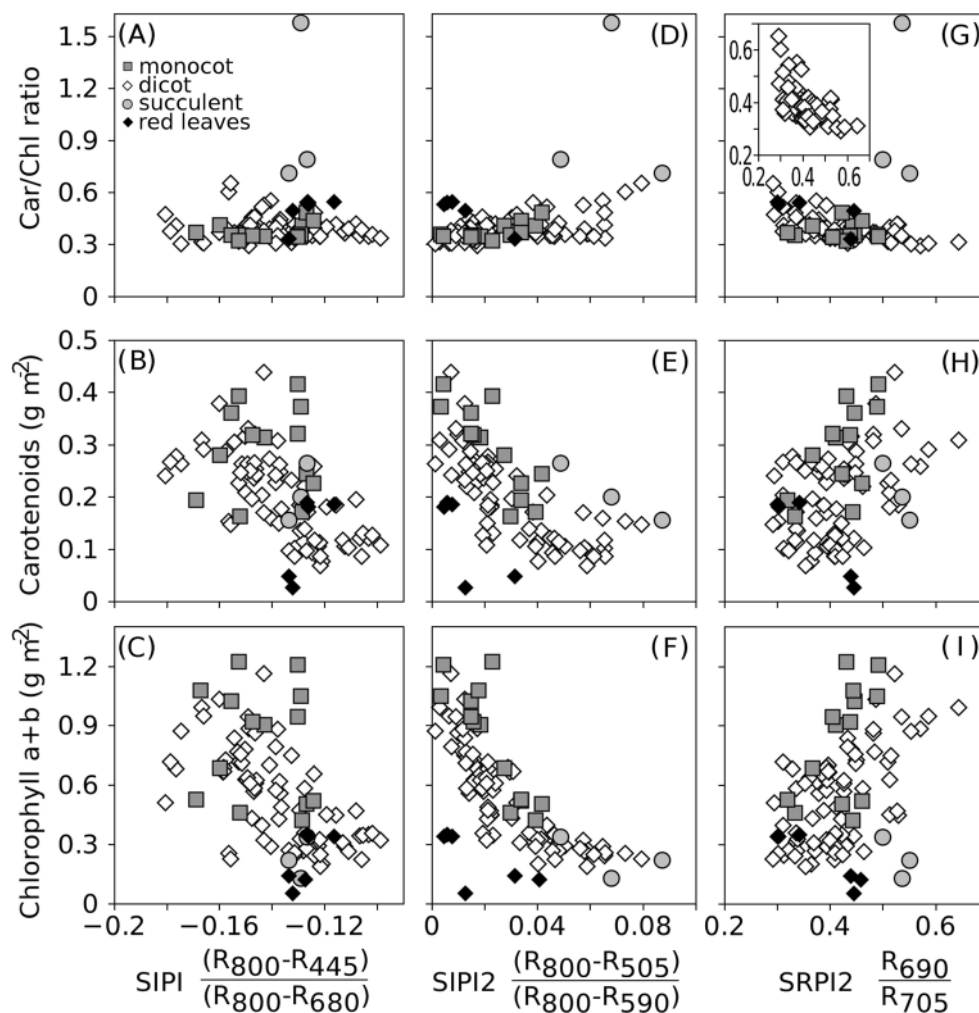
In addition to the bulk of the data consisting of green healthy high-light-exposed leaves, our dataset included two exceptional classes of leaves with markedly different pigment composition: (1) red leaves with high anthocyanin content and (2) succulent leaves (*A. percarneum*) with high Car/Chl ratio. It is notable that the values of the vegetation index, SIPI = (R800 – R445)/(R800 – R680), by Peñuelas et al. (1995) which utilizes spectral bands near the chlorophyll absorption maxima, were virtually identical for both exceptional classes of leaves—red leaves and succulent leaves (Fig. 3a–c). Modification of SIPI by using wavelengths 505 and 590 nm made this index more sensitive to chlorophyll content and clearly separated the red leaves with high anthocyanin content (low SIPI2 index values) from *A. percarneum* (high Car/Chl ratio and higher values of SIPI2) leaves (Fig. 3d–f).

The simple ratio pigment index (SRPI2) that uses the far-red spectral region R690/R705 estimated Car/Chl ratio relatively well in green leaves of dicots (Fig. 3g inset), except for the succulent species *A. percarneum* which was a complete outlier. This far-red spectral region is not corresponding to the known absorption features of carotenoids but should be dominated by chlorophyll *a*. All these results suggest that confounding effects of other accompanying pigments are highly variable across the visible spectrum and avoiding saturation by selecting wavelengths outside of the absorption maximum of the given pigment class does change the sensitivity of vegetation indices in a much more complex way than the simple saturation effect.

### The effect of pigments on leaf spectral properties across 400- to 900-nm region

To illustrate the relationship between leaf pigment content and spectral properties in detail, six different types of vegetation index formulations were calculated across the whole visible to near-infrared spectral range: simple difference (SD), simple ratio (SR), normalized difference (ND), derivative difference (DD), derivative ratio (DR), and normalized derivative difference (NDD) (Fig. 4 and Appendix 3). In this analysis,

**Fig. 3** Vegetation indices utilizing spectral regions with strong absorption by pigments (blue and red) and weaker absorption (green and far-red spectral region) predicting leaf carotenoids to chlorophyll ratio, leaf total chlorophyll content, and leaf total carotenoid contents. Separate symbols denote the monocotyledons (squares) and dicotyledons (rhombs). The only succulent species in dataset (*Aeonium percarneum*) is marked with circles, and visually red-colored leaves collected extra due to higher anthocyanin content are marked with black rhombs



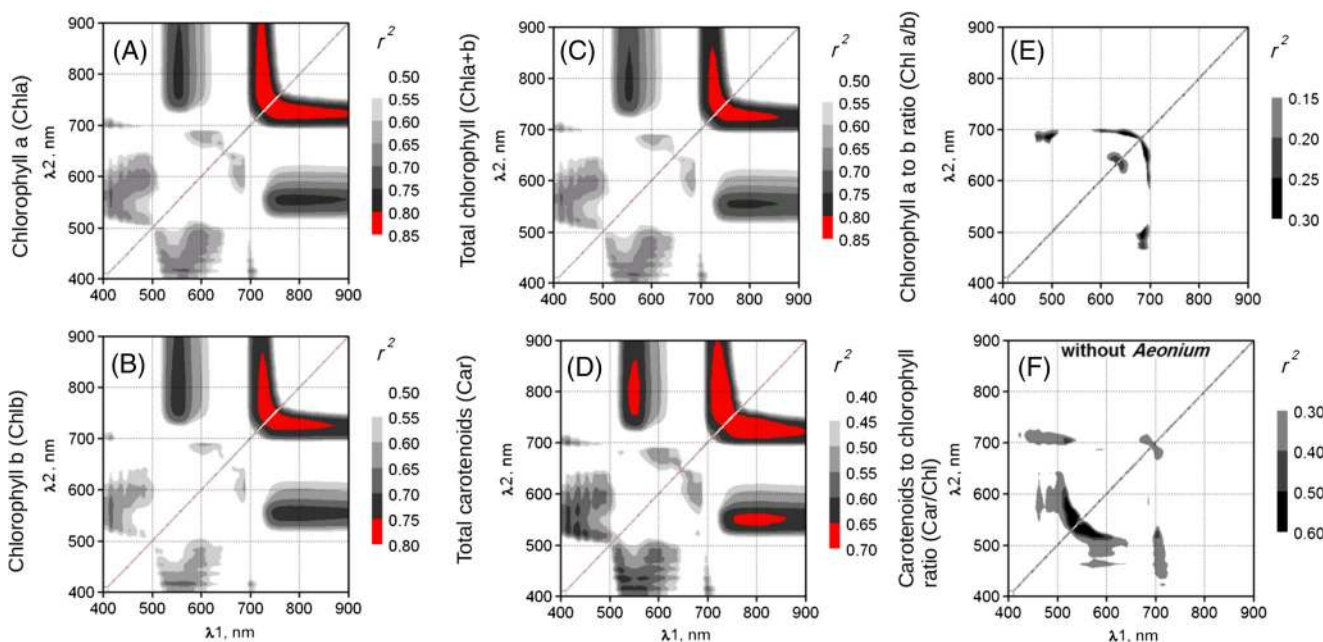
correlations between vegetation indices and pigment contents were tested at each wavelength combination across full 400- to 900-nm spectral region. The best spectral region for estimating chlorophyll content is located beyond 700 nm at the beginning of NIR plateau (Fig. 4). For the best-performing indices, both the “reference” and the “sensitive” wavelengths were in close proximity (Fig. 5, Table 2, and Appendix 3).

Due to strong coordination of the contents of individual pigments and different pigment classes (Fig. 2), the same wavelength regions correlated to leaf chlorophyll contents also predicted well total carotenoid content (Fig. 4). To distinguish the individual effects of different pigments to leaf reflectance from confounding effects of covariation among pigments, we assessed which pigment class had the strongest correlation with given vegetation index. In this step, the vegetation index was considered sensitive to the content of the given pigment class if the correlation with given pigment or pigment class was stronger than with any other pigments or pigment class tested (Appendix 3; pigment-specific maximum correlation). The best spectral region for estimation of Chl *a* still remained beyond the red edge at the beginning of NIR

plateau, but the best indices which are more sensitive to Chl *b* than to Chl *a* were mainly located in the red spectral region between 610 and 690 nm (Appendix 3). In accordance with the known absorbance spectra of carotenoids in solvents, total carotenoid pool was primarily influenced by certain wavelengths in green-yellow spectral region (Appendix 3). However, the correlations with spectral indices sensitive to the content of given pigment or pigment class (other than Chl *a*) turned out to be notably weaker than indirect correlations with wavelength regions dominated by the influence of Chl *a* (Appendix 3).

The possibility of assessing Chl *a* to Chl *b* ratio from reflectance data in the case of high-light-grown foliage of multiple species appears to be modest. The best correlation between vegetation index and Chl *a/b* ratio was  $R^2 = 0.39$ , utilizing wavelengths at 655 and 689 nm (Appendix 4B). Prediction of total carotenoid to total chlorophyll ratio (Car/Chl) from reflectance spectra of green healthy leaves was more accurate. Simple ratio of reflectance at wavelengths of 565 and 526 nm described 66% of Car/Chl ratio if the succulent species was excluded (Appendices 3 and 4A). The





**Fig. 4** Normalized difference indices  $(R_{\lambda 1} - R_{\lambda 2}) / (R_{\lambda 1} + R_{\lambda 2})$  predicting **a** leaf chlorophyll *a*, **b** chlorophyll *b*, **c** leaf total chlorophyll, **d** leaf total carotenoid contents, **e** chlorophyll *a* to *b* ratio, and **f** carotenoids to chlorophyll ratio from leaf reflectance

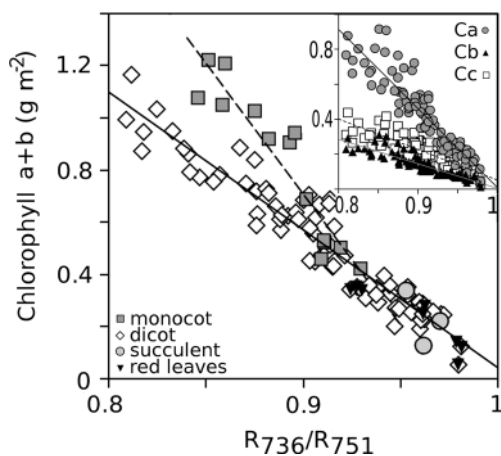
across whole visible spectral region and near-infrared plateau (400–900 nm). Grey levels denote the R-square values of linear relationship between vegetation indices (VIs) and leaf pigments

wavelengths which showed the strongest relationships with Car and Car/Chl in conditioned data (Appendix 3) matched well the known absorption features of carotenoids and chlorophylls in solvents. However, the performance of vegetation indices using this spectral region is strongly affected by the

presence of anthocyanins as well as species-specific differences (Fig. 6).

### Differences between functional groups

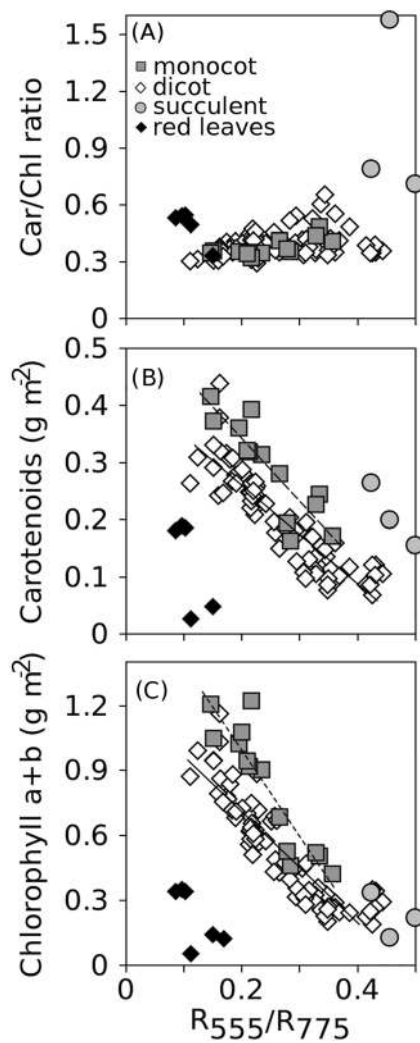
There was no systematic difference between leaf spectral properties of woody and herbaceous plants. Somewhat more surprisingly, different regression formulae were not needed for estimating pigment content of deciduous and evergreen leaves despite of structural differences associated with leaf longevity. The strongest difference was observed between the leaves of monocots and dicots. In particular, the regression slopes to estimate pigment content from reflectance data differed often remarkably between monocots and dicots (Appendix 1, Table 2, and Fig. 5).



**Fig. 5** Simple ratio of reflectance at near-infrared wavelengths 736 and 751 nm predicting leaf total chlorophyll content (Chl *a* + *b*). Separate regressions are shown for monocotyledons (gray squares; regression formula  $y = -10.03x + 9.74$   $R^2 = 0.87$ ) and dicotyledons (rhombs;  $y = -5.31x + 5.35$ ,  $R^2 = 0.92$ ). Inset shows the pigments separately: chlorophyll *a* (Ca; gray circles;  $y = -4.51x + 4.52$ ,  $R^2 = 0.85$ ), chlorophyll *b* (Cb; black triangles;  $y = -1.40x + 1.40$ ,  $R^2 = 0.80$ ), and total carotenoid content (Cc; empty squares;  $y = -1.75x + 1.80$ ,  $R^2 = 0.72$ ) per unit leaf area  $g/m^2$

### Discussion

Pigment stoichiometry can quickly acclimate to environmental conditions. Both the total pigment pools and proportions relative to each other can profoundly change for example during light acclimation of photosynthetic apparatus (Niinemets et al. 2003; Demmig-Adams and Adams 2006; Hallik et al. 2009, 2012). Our dataset contained only high-light-grown leaves and the variability in pigment composition originates from species-specific differences not from the acclimation to



**Fig. 6** Simple ratio of reflectance at green (555 nm) and near-infrared (775 nm) spectral region estimating leaf total chlorophyll (Chl *a* + *b*) and carotenoid (Car) contents and carotenoids to chlorophyll ratio (Car/Chl). Separate regressions are shown for monocotyledons (*gray squares*) and dicotyledons (*rhombs*). Succulent species *Aeonium percarneum* is marked with *circles* and visually red-colored leaves with higher anthocyanin content are marked with *black rhombs*

different light conditions. Such selection of leaves bears a resemblance to the situation in stand-level remote sensing measurements from satellite platforms where the reflectance signal is dominated by the spectral properties of uppermost leaves of the multispecies canopy. For generalization of our results, we emphasize that all the material originates from plants grown outdoors in Mediterranean conditions and leaves were collected in the beginning of winter, which can also affect pigment composition. The main environmental stress factors plants were experiencing during that period are simultaneous low temperature and high light (Niinemets 2010b), which nevertheless allow the plants to maintain carbon gain in warmer days, especially given that water availability does not limit carbon gain at that period of the growing season (Gulías et al. 2009). Global warming simulations predict a

general water scarcity and an increase of annual mean air temperatures in the Mediterranean region. In Balearic Islands, the annual mean precipitation decreased around 30% between 1951 and 2006. At the end of twenty-first century, a precipitation decrease of around 24% is expected, and a change of the precipitation pattern, presenting longer drought periods and falling most rainfalls during winter months. The mean annual temperature during 1900–2000 has increased 0.46 °C (Llites et al. 2012). Relationships between vegetation indices and leaf pigments allow to monitor vegetation responses to such environmental changes via optical remote sensing measurements.

Different leaf structures as sclereids, leaf bundle sheath extensions, cell walls, and mesophyll cells have been described as traits affecting the spectral properties of leaves (Poulson and Vogelmann 1990; Smith et al. 1997; Nikolopoulos et al. 2002). We found the strongest difference between monocot and dicot leaves which required separate regression slopes for accurate estimation of pigment contents from leaf reflectance. Species classified as monocot and dicot differ in their leaf structure, showing main contrast on mesophyll differentiation and distribution of venation. Mesophyll structures of leaves influence the penetration of light (Vogelmann and Martin 1993). Monocots present basically only spongy mesophyll, which is known to scatter light and increase the light absorption per unit of chlorophyll (Terashima and Saeki 1985; Bornman et al. 1991). On the other hand, dicots present a clear differentiation between spongy and palisade mesophyll. In that case, besides spongy tissue that favors light scattering, palisade cells canalize light into lower mesophyll layers (Vogelmann et al. 1996; Smith et al. 1997). Moreover, the leaf thickness influences in the number of cells per leaf area, and consequently, the chlorophyll content (Tosens et al. 2012).

While acclimation processes in general can seriously affect the relative share of different pigment pools (Hallik et al. 2012; Esteban et al. 2015a), in our dataset, Chl *a*, Chl *b*, and Car pools are tightly intercorrelated as we focused on upper canopy leaves grown at high-light availability. The finding that wavelength regions primarily sensitive to Chl *a* turned out also as the best predictors for Chl *b*, Chl *a* + *b*, and Car could be explained simply by the pigment pool sizes. These leaves contained roughly three times more Chl *a* than Chl *b* or carotenoids. However, the spectral region which proved to be the best predictor of Chl *a* and other pigments was located at much longer wavelengths than PSI reaction center absorption at 700 nm. Mechanistically, light should be absorbed in this spectral region by the small number of specific pigment-protein complexes, so called “far-red chlorophylls” (Gobets and van Grondelle 2001; Melkozernov 2001; Gibasiewicz et al. 2005), which nevertheless could transfer light energy to both PSII and PSI (Oja et al. 2004; Pettai et al. 2005a,b). The existence of such pigments has been shown and

investigated in plant physiology studies dealing with fine structures and functions of photosynthetic apparatus (e.g., Rivadossi et al. 1999), but this knowledge has not yet reached out to wider community of remote sensing and ecosystem-level research. Considering the significance of red-edge spectral region for vegetation remote sensing studies, the questions such as the effect of PSI to PSII ratio on remote sensing estimates of vegetation properties may also deserve more attention than it has received until now. Although in remote sensing studies of leaf optics, NIR plateau is traditionally considered as the reference region with no influence of pigments, subtle changes in leaf transmittance at 820 nm are commonly measured to investigate the performance of PSI in plant physiology studies (Oja et al. 2003). PSI complexes possess absorption and emission bands at lower energy than those of the reaction center (700 nm) (Ihalainen et al. 2003). Some studies suggest that extreme long-wavelength chlorophylls may be also present in the PSII antenna system (Zucchelli et al. 1990; Pettai et al. 2005a,b; Thapper et al. 2009). Long-wavelength quanta up to 780 nm can support oxygen evolution from the leaves, and the quantum yield of oxygen evolution at the local maximum at 745 nm reaches almost 20% of the yield at 650 nm, with possible existence of a small number of far-red chlorophylls of photosystem II (Pettai et al. 2005a, b; Thapper et al. 2009).

Sentinel-2 MSI has several spectral bands in red-edge and NIR spectral regions which allow to calculate VIs which performed well in our dataset. Recent study by Kira et al. (2015) showed that more complicated multispectral methods provide only modest improvement compared to simple VIs for estimating pigment content. As their dataset contained only deciduous leaves and pigment content variability was created by phenology, it is not surprising that some wavelength regions suggested by Kira et al. (2015) do not estimate pigment content well in our dataset. Evergreen leaves in our dataset have two times higher pigment content compared to the dataset used by Kira et al. (2015). Still both datasets agree in good performance of VIs using wavelength regions beyond 700 nm.

Chlorophyll content is one of remotely sensed essential biodiversity variables (RS-EBVs) (Skidmore et al. 2015; Pettorelli et al. 2016). It has been shown that monitoring the state of biodiversity and ecosystems at national scale can benefit from the concept of RS-EBVs (Vihervaara et al. 2017). Estimation of canopy cover, leaf area index, and greenness phenology from optical remote sensing rely all mechanistically on light absorption in leaf pigments. There is also a strong correlation between chlorophyll and nitrogen content in foliage. Remote sensing estimation of chlorophyll content can be used for vegetation health monitoring, forage quality assessment, biomass estimation, productivity measures, etc. Leaf chlorophyll content multiplied with LAI gives total canopy chlorophyll content.

It can be concluded that due to strong coordination of different pigments in leaves, wavelength region selection for estimating any single pigment class is strongly affected by the interrelationships between different pigment pools. In particular, the empirical relationships between vegetation indices and other pigments tend to be dominated by the effect of chlorophyll *a*. The “red-edge” spectral region and the beginning of NIR plateau at wavelengths notably larger than 700 nm deserves more attention and is still sensitive to chlorophyll content up to 730–740 nm. One explanation for good predictive power of this spectral region could be that confounding effects of defense mechanisms against excess absorption of light do not operate in wavelength region beyond 700 nm. The major structural effect to consider in prediction formulae of leaf pigment contents appeared to be the different leaf anatomy of monocots and dicots.

**Acknowledgements** This study has been supported by a travel grant between the Estonian Academy of Science and Consejo Superior de Investigaciones Científicas (CSIC), the Estonian Ministry of Science and Education (institutional grant IUT-8-3), by the targeted financing of the Ministry of Education and Research, Project SF0180009Bs11, and Project AGL2009-07999 (Plan Nacional, Spain). This project has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement no. 687320 and the European Commission through the European Regional Fund (Centers of Excellence ENVIRON and EcolChange), Estonian Research Mobility Scheme ERMOS73, European Social Fund program Mobilias grant MJD122, and COST Action ES1309. We would like to thank Jardí Botànic de Sóller and Mr. Miquel Truyols of the UIB Experimental Field and Greenhouses (UIB Grant 15/2015).

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