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Title: Quantification of chlorophyll and carotenoid pigments in eucalyptus foliage with the radiative transfer model PROSPECT 5 is affected by anthocyanin and epicuticular waxes.

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Abstract

Precision monitoring of plant health via remote sensing benefits from accurate quantification of attributes such as foliar pigments related to stress. For example, pigment content (the ratio of chlorophyll to carotenoids) can be indirectly related to photosynthetic efficiency in plants and hence productivity. Optical remote sensing can potentially provide this data with high spatial accuracy via spectral reflectance. In a previous study we found that the leaf radiative transfer model PROSPECT 5 could estimate chlorophyll content in *Eucalyptus globulus* leaves reasonably well but prediction of carotenoids was poor. To examine the basis for poor model performance, we conducted a targeted study using both a pot experiment with *E. globulus* and field samples of *E. globulus* and *E. nitens*. We examined the influence of anthocyanins and waxes on the performance of the model by manipulating these variables with shade, nutrition and a wax removal treatment. The relationship between measured to modelled leaf chlorophyll concentration was strongest when plants were shaded then exposed to several days sun before assessment, (r^2=0.88), in which anthocyanins were 3-fold lower than the “sun” treatments. The fit was also improved for “sun” treatment plants when wax was removed from leaves (r^2=0.77). This highlights the impact of anthocyanins and wax on performance of PROSPECT5 to predict chlorophyll in eucalypts. Surprisingly, the fit between measured and modelled carotenoid concentrations was not greatly improved by any of the treatments - the best fit achieved was for the treatment with wax removed (r^2=0.42). For the adult leaves collected in the field, we investigated the impact of anthocyanin content on the fit of measured to modeled carotenoids, but results were not greatly improved when using only leaves with low anthocyanin content. During this study we refined our UV spectroscopy methods for both chlorophyll and carotenoid estimation, and these refinements were validated by analysis of the same samples with ultra-high performance liquid chromatography. After method refinement, data generated from the two methods were closely related (carotenoids, r^2=0.99; chlorophyll, r^2=0.97) and method problems were not contributing to poor relationships between measured pigments and those modelled by PROSPECT5. We conclude that the presence of waxes in eucalypts leaves is the main source of error in the estimation of pigment concentrations using PROSPECT 5. These results suggest that application of PROSPECT 5 to eucalypt canopies across different seasons may be problematic, particularly for quantification of carotenoids, as both anthocyanins and waxes can change with season, ontology and stress. However, these could be accounted for in future versions of the model.

Key words

Eucalyptus, foliar pigments, PROSPECT, stress detection

Author biography

K.M. Barry; Karen is a lecturer in Plant Pathology and has conducted research in plant health since completing her PhD in 2001. This has included disease management, detection of abiotic and biotic stress and impact of damage on growth. She has experience in plantation forestry and horticulture. She is particularly interested in applications of remote sensing for detection of plant disease.

G. J. Newnham; Glenn is a senior research scientist with the CSIRO with 10 years experience in land surface remote sensing. His key research areas include passive short wave sensing of vegetation condition and the use of ground and airborne lidar sensing of vegetation structure.

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Introduction

Eucalyptus globulus is an important plantation species in Australia and forest health assessment is an expensive component of management. Remote sensing has the potential to offer spatially explicit information on forest condition. Remote sensing in the short-wave domain is based on spectral reflectance detected by spectroradiometers, which may be hand-held, aircraft or satellite-borne. Spectral reflectance of vegetation is a result of absorption of foliar components between 300-2500 nm, which ranges from pigments, waxes, water, cellulose, lignin and structural features such as cell size.

The ratio of chlorophyll and carotenoid pigment content is strongly related to photosynthetic functioning of vegetation (Gamon *et al*., 1997) and this capacity varies in a range of environmental conditions. Periods of medium- to long-term stress can often be detected by decreases in chlorophyll content (Lichtenthaler 1996; Zarco-Tejada *et al.* 2002), while short-term changes can be more easily detected via carotenoid metabolism (Demmig-Adams and Adams 1996). Methods to accurately detect changes in chlorophyll content in agricultural and forest landscapes via remote sensing have been developed using spectral indices (simple equations using at least two wavelengths) (Coops *et al.* 2003; Datt 1998), radiative transfer models which can be used with hyperspectral or multispectral imagery (Jacquemoud and Baret 1990) and fluorescent approaches (Zarco-Tejada *et al.* 2002). Carotenoid content has been estimated only by spectral indices (Gitelson, 2002) until new developments in radiative transfer models recently (Feret *et al*., 2008). Our recent research has shown that while chlorophyll content can be determined in eucalypts very well with spectral indices (Barry *et al*., 2008; Barry and Mohammed 2008), carotenoids have been very poorly estimated (Barry and Mohammed 2008).

Leaf-level radiative transfer models such as PROSPECT (Jacquemoud and Baret 1990) can be coupled to canopy models such as SAIL (Jacquemoud et al., 2008) to enable accurate quantification of chlorophyll content via remote sensing, as factors including foliage and tree structure, background and solar angles can be taken into account in the models. This provides advantages compared to vegetation indices. PROSPECT version 3 and 4 (P3 and P4) have been designed to predict four key leaf variables (water, dry matter, chlorophyll and leaf structure) from reflectance and transmittance spectra (obtained with a spectrophotometer coupled with an integrating sphere to enable capture of all transmitted light), and can be coupled with canopy models to obtain estimates of these variables across canopies (Jacquemond *et al.* 2008). Leaf-level studies of plantation eucalypts have shown that both versions of PROSPECT provide good predictions of total chlorophyll in *E. globulus* leaves, as either juvenile or adult morphology (Barry *et al*., 2009). P4 reduced the error associated with estimated total chlorophyll to around 5 µg cm^{-2} for juvenile leaves and 3 µg cm^{-2} for adult leaves (Barry et al., 2009). These results are an improvement on multiple species studies and other single species studies using P3. For example, Renzullo *et al* (2006) obtained ± 10 µg cm^{-2} for grapevine of different varieties using the same P3 version as here, although only reflectance was used. Feret *et al.* (2008) found that across data sets with a wide range of plant genera, an average of ± 9 µg cm^{-2} was obtained with P4.

The development of PROSPECT 5 incorporated estimation of carotenoid pigments into the model (Feret *et al*., 2008). Preliminary application of PROSPECT 5 to eucalypt foliage resulted in poor carotenoid estimations (Feret, J-B and Barry, K, unpublished), although when included in a broad data set of various genera this was not detectable (Feret *et al*., 2011). In addition, our attempts to apply spectral indices designed for estimation of foliar carotenoids have yielded poor results (Barry, K, unpublished). We propose that this may be because the spectral indices tested have been developed with data sets of foliage with little anthocyanin content (a red "photoprotective" pigment) and no surface wax (Gitelson *et al*., 2002). Eucalypt leaves of all ages typically have high anthocyanin content and juvenile leaves have high epicuticular wax content.

To investigate the possible role of anthocyanin and waxes on the ability of spectral methods to estimate carotenoids, we conducted field and pot experiments with *E. globulus* and *E. nitens*. Foliage with a range of anthocyanin and wax content was manipulated with nutrition and shade treatments in the pot trial, while foliage of different ages was utilised in the field grown samples.

Materials and methods

Pot study
E. globulus (blue gum) were grown from seedlings and 32 plants were potted into 15 cm diameter pots. Plants were fertiliser with Osmocote for natives, a long release fertiliser. Half the plants (16) were maintained in a shadehouse while the other 16 were maintained in an unshaded outdoor enclosure. Adequate water was provided to plants at all times.
Four "treatments" were established;
1) SUN – Plants maintained in the sun,
2) SUN_WAX - Plants maintained in the sun, with epicuticular wax gently removed from target leaves on the day of sampling,
3) SHADE – Plants maintained in the shade,
4) SHADE_SUN – Plants maintained in the shade until 24 hours before sampling, when they were moved in the sun.

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Therefore 8 plants per treatment were used for the study. Half of the plants from each treatment (4) were randomly chosen to be sampled on day 1 while the other half were chosen for sampling on day 2. Plant height and general condition were recorded on the day of sampling.

Field study

E. nitens (shining gum) leaves were sampled from a commercial plantation near Buckland, southern Tasmania. *E. globulus* were sampled from a garden in Sandy Bay, southern Tasmania. In each case, 3 leaves from each of 3 branches of each of 3 trees were chosen for sampling (27 leaves each per species). Leaves were all of adult morphology, but a range of younger to more developed leaves were selected to obtain a range of pigment concentrations. Sampled leaves were placed in labelled plastic bags in an eski and returned to the laboratory. Spectral measurements and leaf sampling were conducted the same day, as described below.

Spectral reflectance measurements

Spectral reflectance was determined within 6 hours of sampling for the field grown plants (leaves were maintained on ice) and within 10 min for the pot grown plants. An Analytical Spectral Devices FieldSpec® Pro FR spectroradiometer (ASD, Boulder, USA) was used with a LiCor integrating sphere attached to collect stable bi-hemispherical reflectance measurements. Due to low spectral intensity of the halogen lamp used for the integrating sphere above 1600 nm and the resulting noise in the measured spectra, reflectance and transmittance was only considered below this wavelength. Reflectance and transmittance were recorded from the same leaf position on one side of the mid-vein. Spectra were corrected for stray light by subtraction from both the transmittance and reflectance data, and a conversion factor between the barium sulphate surface of the integrating sphere and a Spectralon® reference panel.

Foliar properties and pigments

Immediately following spectral data collection, leaves were sampled for destructive analysis. The side of the leaf opposite to that used for spectral assessment was retained for pigment analysis and 4 leaf discs were immediately stored in liquid nitrogen. Two leaf discs (0.50 cm^2 each) were removed from the other side of the leaf, a fresh weight obtained and then they were stored for oven drying to obtain leaf water content (*Cw*) and leaf dry matter (*Cm*).

Two of the frozen discs were extracted for chlorophyll and carotenoid content with a triple extraction method (Martin *et al.* 2007). Discs were ground in a mortar with approximately 50 mg $MgCO_3$, 50 mg washed, fine sand and a small volume of liquid nitrogen. Ground leaf material was extracted with three small volumes of 100% cold acetone, centrifuged for 3 minutes then absorbance was read with 470, 645, 663 and 710 nm with a Cary UV-VIS spectrophotometer. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids (TCar) were calculated using the equations of Lichtenthaler and Buschmann (2001). Total chlorophyll (Chl *a*+*b*) and the ratio of Chl *a* to Chl *b* was determined (Chl *a/b*).

In addition to analysis via UV-VIS spectrophotometry, some samples were further analysed by ultra-high performance liquid chromatography (UPLC) to quantify xanthophyll pigments (violaxanthin, antheraxanthin and zeaxanthin, neoxantin and lutein), β-carotene and chlorophyll *a* and *b*. UPLC was performed with a Waters Alliance 2690 (MA, USA) and Waters 996 Photodiode Array Detector. The column was a non-endcapped Spherisorb ODS-1, 250 x 4.6 mm ID (Alltech, Deerfield, IL, USA) which was used with an ODS-1 guard column. Total carotenoids (*TCar*) were determined as the sum of xanthophyll pigments (zeaxanthin, antheraxanthin and violaxanthin) and β-carotene. Pigment results for both methods (UV-VIS and UPLC) of quantification for chlorophyll and carotenoids were compared to test if they were in agreement. We found a strong relationship between both methods (r^2=0.97 and 0.99 respectively), therefore we believe that quantification of pigments with our methods is highly accurate.

Anthocyanins were extracted with acidified methanol (methanol:water:HCl, 90:1:1) using the same triple extraction of ground discs as described above for chlorophyll and carotenoids. Extracts were centrifuged and absorbance read at 530 and 657 nm. Total anthocyanin content was calculated using these absorbance measurements in a published equation (Mancinelli *et al.* 1975) and converted to molar concentration using a molar absorbance coefficient of 30,000 L mol^{-1}.

PROSPECT 5 modelling

The PROSPECT 5 radiative transfer model was used as described in Feret et al. (2008). Using measured reflectance (R_{meas}) and transmittance (T_{meas}) spectra from 400-2500 nm, PROSPECT model estimates of total chlorophyll (*Cab*, µm cm^{-2}), total carotenoids (*Car*), leaf water content (*Cw*), dry matter content (*Cm*) and mesophyll structure parameter (*N*) were derived using inversion. The merit function for the inversion is shown in Equation 1, where the subscript mod refers to modelled estimates of reflectance and transmittance. The derived estimates were then compared to measured values of *Cab*, *Car*, *Cw* and *Cm*. Estimates of *N* could not be validated since this parameter described the scattering power of the leaf internals material and is only indirectly associated with physical structure.

$$\chi^2 = \Sigma\left[(R_{meas} - R_{\mathrm{mod}})^2 + (T_{meas} - T_{\mathrm{mod}})^2\right] \qquad (1)$$

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Using the measured values for *Cab*, *Car*, *Cw* and *Cm*, and a value of *N* derived from the inversion, modelled reflectance (R_{mod}) and transmittance (T_{mod}) spectra from 400-2500 nm were generated. The accuracy of these modelled spectra was tested against the measured data.

Statistical analysis
All data was checked for assumptions of normality and homoscedasticity with the program SPSS (Version 13) and log transformed where required. For the pot trial, treatment effects on foliar properties were tested with a general linear model using SPSS. Linear relationships between measured and modelled data were also performed with SPSS.

Results

Foliar properties
There was a statistically significant effect of treatment on foliar pigments of *E. globulus* pot grown plants (Table 1). Plants exposed to sun throughout the treatment period had the highest content of anthocyanin and lower chlorophyll (Table 1). Throughout the whole data set, anthocyanin content ranged from 0.0 - 21.5 nmoles/cm^2, chlorophyll from 10.9 - 48.3 µg/cm^2, and total carotenoids from 5.2 – 11.2 µg/cm^2. Foliar pigment content for adult leaves from mature, field-grown *E. nitens* and *E. globulus* were substantially higher than the pot-grown plants (Table 2). Foliar *Cw* and *Cm* were determined, as they are essential input parameters for the PROSPECT model (data not shown).

Table 1. Foliar properties of pot-grown E. globulus *plants subjected to different light exposure or wax removal treatments.* SUN – Plants maintained in the sun; SUN_WAX - Plants maintained in the sun, with epicuticular wax gently removed from target leaves on the day of sampling; SHADE – Plants maintained in the shade; SHADE_SUN – Plants maintained in the shade until 24 hours before sampling, when they were moved in the sun.

Variable	SUN	SUN_WAX	SHADE	SHADE_SUN	P value
Chl ab (µg/cm^2)	27.2 ± 1.6^{a}	29.3 ± 1.8^{ab}	33.2 ± 5.9^{b}	32.3 ± 1.1^{b}	0.015
TCar (µg/cm^2)	7.9 ± 0.4^{b}	8.5 ± 0.3^{bc}	7.3 ± 1.1^{ab}	7.2 ± 0.3^{ab}	0.014
Anth (nmoles /cm^2)	3.7 ± 0.9^{a}	2.4 ± 0.9^{ab}	1.0 ± 0.2^{bc}	0.5 ± 0.1^{c}	0.003

Table 2. Foliar properties of field grown E. nitens *and* E. globulus *trees.*

Variable	*E. nitens*	*E. globulus*
Chl ab (µg/cm^2)	52.3 ± 1.9	77.4 ± 2.4
TCar (µg/cm^2)	12.9 ± 0.5	24.2 ± 0.6
Anth (nmoles /cm^2)	5.8 ± 0.6	1.1 ± 0.8

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Effect of wax and anthocyanin on spectral properties

The reflectance and transmittance spectra of leaves from pot trial *E. globulus* plants were characteristic of healthy green vegetation (Fig. 1). Leaves with low anthocyanin content had much higher reflectance in the visible wavelengths (400-650 nm) than transmittance and a defined “green peak” at 550 nm (Fig. 1A). However when anthocyanin was approx. 4 fold higher (but chlorophyll and carotenoids at similar levels), the green peak was less defined (Fig. 1B). Representative spectra of *E. globulus* leaves with (Fig. 1C) and without wax show that removal of wax led to spectra with less reflectance in the visible wavelengths (Fig. 1D).

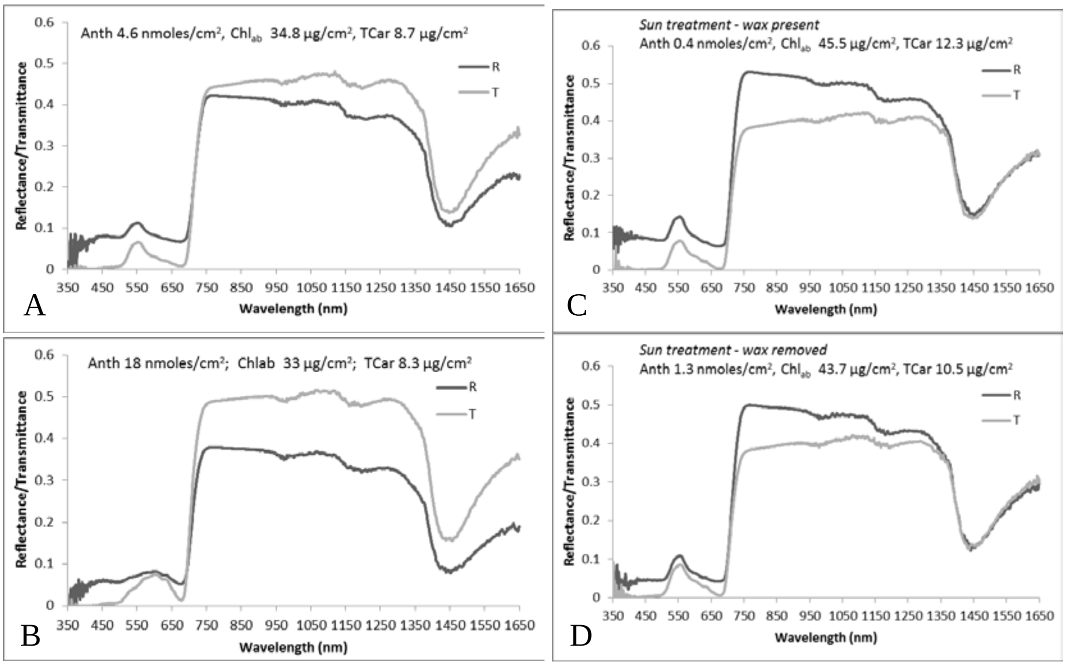

Figure 1. Representative reflectance and transmittance spectra for selected foliar samples of potted E. globulus plants used in the study. A) an adult leaf with low anthocyanin content, B) an adult leaf with high anthocyanin content, C) a juvenile leaf with low anthocyanin and wax present, D) a juvenile leaf with low anthocyanin and wax removed.

Performance of PROSPECT 5 for foliar pigments

Across the whole data set, a highly significant and strong relationship between measured and modelled *Cab* was found (Table 3) reflecting good model performance of PROSPECT 5 for prediction of this pigment. Performance was particularly poor for the *E. nitens* adult leaves, which was unexpected (Table 3). The leaves collected from this species included several young leaves of red appearance, and the sample set had much higher average anthocyanin content than *E. globulus* (Table 2). Model performance for the pot trial data was generally good with lower RMSE than the adult field samples (Table 3). The best model fit for Cab was for foliage from plants which were shaded and then sun exposed prior to assessment (Table 3), which had the lowest average anthocyanin content of all treatments. Therefore, all these data sets suggest that there may be an influence of anthocyanin content on the ability of P5 to estimate chlorophyll content. Removal of wax also improved the estimation of Cab by P5 (Table 3). Estimation of *TCar* by P5 for this data set was generally poor (Table 3), particularly for the field samples. The best estimation of *TCar* was for foliage from the sun-exposed pot plants with wax removed (Table 3) which highlights the role that wax has in the spectral properties of eucalypt leaves.

Table 3. Linear relationship between measured and modelled values of foliar pigments using P5, reporting the adjusted R^2, P value (ANOVA) and root mean square error (RMSE).

		Cab			*TCar*		
	n	R^2	P	RMSE	R^2	P	RMSE
All samples (n=140)	140	0.86	0.000	7.88	0.27	0.000	5.39
E. nitens - field samples	26	0.42	0.001	10.1	0.18	0.037	2.37
E. globulus – field samples	18	0.68	0.000	7.61	0	NS	NA
E. globulus – pot-trial	96	0.78	0.000	3.21	0.22	0.000	1.37
Sun	24	0.67	0.000	2.81	0.35	0.001	0.78
Sun_wax	24	0.77	0.000	2.83	0.42	0.000	0.82
Shade	24	0.77	0.000	3.65	0	NS	NA
Shade_sun	24	0.88	0.000	2.96	0.23	0.012	1.47

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Discussion

This study highlights that use of PROSPECT5 for estimation of carotenoids in the eucalyptus species tested is currently unreliable. We propose that this could be rectified by inclusion of anthocyanins and wax parameters in the model. This would require absorption coefficients for these components as pure compounds, which while available for anthocyanins, is not yet available for waxes. Even with an absorption coefficient the possible specular reflectance of wax may require more in depth study so that it can be modelled adequately in PROSPECT. i.e. leaf bidirectional reflectance. This presents a potential research opportunity.

Estimation of carotenoids is of particular interest based on their role as indicators of stress and photosynthetic efficiency (Gamon *et al*., 1997; Lichtenthaler 1996; Demmig-Adams and Adams 1996). While estimation of total quantity is desirable, other options to indicate plant stress include estimating the epoxidation state (EPS) of xanthophyll pigments, upon which the photochemical reflectance index (PRI) (Gamon *et al.* 1997) is based. The PRI can also be correlated to light use efficiency. These relationships can be explored with the pot trial data set from this study to some degree, as EPS of xanthophylls from pot trial samples in the current study was determined, as was light use efficiency of the leaves before they were removed from the plants. Recent studies have also highlighted that the PRI can be used to better estimate carotenoid content, if chlorophyll content is accounted for (Garrity *et al*., 2011).

While estimation of carotenoids was poor, the estimation of chlorophyll content with P5 was adequate and considered reliable because independent validation data was used that is not reliant on carotenoid accuracy. While there is some overlap in the spectral absorbance region for both pigment types, carotenoid absorption only occurs in the blue/green wavelengths, while chlorophyll absorption occurs in both the blue/green and red wavelengths.

Conclusion

Performance of PROSPECT 5 for chlorophyll estimation was adequate for the samples tested in this study, however it was poor for carotenoid estimation. Our pigment analysis methods were shown to be highly accurate and therefore we conclude that pigment quantification problems do not contribute to issues with P5 model performance in this study. We demonstrated the influence of both anthocyanins and wax on P5 model performance and suggest that these factors need to be included in future models to attain high performance for eucalyptus species.

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