MONITORING WATER POTENTIAL AND RELATIVE WATER CONTENT IN *EUCALYPTUS CAMALDULENSIS* USING NEAR INFRARED SPECTROSCOPY

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A genetically diverse cohort of *Eucalyptus camaldulensis* seedlings were grown under a range of irrigation treatments to impose a range of states of water stress. Near infrared (NIR) spectra obtained from leaf surfaces of these seedlings were calibrated against physiological measurements commonly associated with plant water stress, i.e. relative water content and midday leaf water potential. Spectral data were obtained from upper (adaxial) leaf surfaces of upper and lower leaves on the stem. A strong coefficient of determination of $R^2_P = 0.78$ was obtained for the validation set using the calibration developed for midday leaf water potential from the seedling leaf data. However, that for relative water content was relatively low: $R^2_P = 0.41$. These results indicated that portable NIR has the potential to provide for rapid, non-destructive assessment of the leaf water potential in *E. camaldulensis* seedlings.

Keywords: Physiology, portable NIR, non-destructive assessment

INTRODUCTION

Eucalyptus species are among the most important fast-growing, high-yielding forest plantation species in China, with the area planted to such species now being well in excess of 4.0 Mha. As raw material feedstock for production of pulp, wood panels and timber, their plantations provide the foundation for world-class wood industries which offer employment and improved livelihoods to a great many Chinese (Luo et al. 2012, Arnold et al. 2013).

Rapid expansion of eucalypt plantations in China over the past 30 years can be attributed to a combination of many factors including enhancements in plantation productivity, and hence profitability, provided by both genetic and silvicultural advancements (Barr & Cossalter 2004, Turnbull 2007). However, achieving further productivity improvements and ensuring future sustainability of eucalypt plantations in China is fraught with increasing challenges and threats, including limitations on water availability and a number of factors related to changing climates.

In China, *Eucalyptus camaldulensis* is of major economic importance to commercial eucalypt growers, being valued as a parent of hybrid taxa from which some of the more commonly planted commercial *Eucalyptus* clones have been selected (Luo et al. 2014). It is favoured as a hybrid parent in China on account of various economically important traits which it can impart to hybrid progeny, including resistence to lodging in strong winds, vegetative propagation ability and drought tolerance (Luo et al. 2012). In a number of other countries, including South Africa, India, Brazil and Australia, *E. camaldulensis* has also proved useful as a parent of hybrid varieties well adapted to a range of stressful environments, including those with lower rainfall (Little et al. 2003, Barbour 2004, Potts & Dungey 2004). Drought tolerance is of key economic importance for commercial plantation eucalypts in drought susceptible environments, as it affects plantation productivity during periods of limited precipitation and soil moisture availability.

Physiological traits related with plant and tree water status, especially relative water content and leaf water potential are often studied to understand and assess variation in drought resistance in tree species (Turner 1981, Ngugi et al. 2004, White et al. 2016). However, conventional approaches for the measurement of such traits can be time-consuming, costly and also destructive, limiting applications in genetic, longitudinal and other studies (Warburton et al. 2014). In order to evaluate genetic variation in such traits in tree breeding populations, with a view to selection and improvement of these traits, many hundreds or even thousands of individual trees need to be assessed. To do this efficiently, a rapid, economical, non-destructive and accurate technique for measurement of water related traits in trees is required.

Near infrared (NIR) spectroscopy has already proven able to quickly, efficiently and relatively cheaply provide indirect predictions of plant tissue constituents (Foley et al. 1998, Thumm et al. 2010), hybridisation in both soft- and hardwoods (Meder et al. 2014), as well as physiological traits in tree species (Serbin et al. 2012) and a range of agricultural and forage plants (Bei et al. 2011, Cozzolino et al. 2013). Recently, Warburton et al. (2014) reported good success using NIR spectroscopy for the rapid assessment of physiological traits of *E. grandis*, with correlations (R^2's) between actual (traditionally measured) and NIR-estimated measures (made using a portable NIR instrument) of relative water content and leaf water potential of 0.85 and 0.74 respectively.

The objective of this current study was to develop a calibrated model using NIR spectroscopy for assessing physiological traits commonly associated with plant water stress, relative leaf water content and leaf water potential, and to determine whether the methodology can be used to accurately and efficiently estimate such traits in *E. camaldulensis*.

MATERIALS AND METHODS

Genetic material

Seedlings used in this study to provide calibration and prediction (validation) sets of plants were selected randomly from among a subset of seedlings propagated for an *E. camaldulensis* breeding population, comprising seedlots from natural stand seed sources in Australia. Details of the seed sources from which sample seedlings were obtained from are provided by Luo et al. (2014).

The actual genetic identity of the seedlings used in this current study was considered to be of little relevance other than to ensure that the sample group was drawn from a wide genetic base in order to develop a robust NIR calibration, and then to provide good diversity in the calibration set. A previous study involving NIR spectra obtained from leaves of *E. grandis* for calibration against physiological measurements, including relative water content and leaf water potential, found no segregation between provenances for such traits (Warburton et al. 2014).

Propagation

The seedlots were sown into special propagation trays containing a germination substrate comprising 60% peat and 40% perlite. At about 4 weeks after sowing, seedlings at the cotyledon plus two leaf pair stage were transplanted into plastic pots of approximate dimensions (height × diameter) of 155 mm × 160 mm, containing media comprising 33% carbonised rice husk, 33% peat and 33% treated coconut dust (coir). A slow release fertiliser (9N:14P:19K + 3MgO + 0.5Fe) had been premixed with the growing media (400 g m^{-3}).

The media in the pots was packed to a bulk density of approximately 1000 kg m^{-3}. After seedlings were transplanted into the pots they were maintained in a naturally lit glasshouse and for the first two months all were provided with identical irrigation.

Irrigation treatments

In November 2014, 420 seedlings of approximately equal size (above ground heights of around 35 cm) were selected. These were randomly divided into the four irrigation treatments (105 seedlings per treatment). A two-day cyclical irrigation treatment regime was applied to all plants, with four irrigation levels following methodology described by Ngugi et al. (2004). In the control treatment (T100) seedlings were watered every two days with 100% of the water lost (from their pots) through evapotranspiration. Water loss was estimated by determining the average weight change of a random sample of five of the T100 pots (over the period since the previous irrigation). In the other four treatments, plants received 70 (T70), 50 (T50) or 30% (T30) of water supplied to control plants.

NIR spectra collection

Sixty days after irrigation treatments commenced, 3 seedlings per irrigation treatment were chosen randomly for obtaining NIR spectra (i.e. 12 seedlings). On each of these seedlings the second pair of fully expanded leaves (downwards from the apex) were scanned on the upper (adaxial) leaf surface using a portable NIR spectrometer. This instrument has 12 nm optical resolution, acquiring 100 spectral data points per scan with a wavelength range of 1600–2400 nm. Three NIR spectra (5 scans per spectrum) were acquired from each of the two leaves of the leaf pair while the leaves were still attached to the seedling stem. These three spectra were then averaged to provide a single NIR spectrum from each individual leaf.

Spectra were only acquired from the adaxial leaf surfaces, as Warburton et al. (2014) found only little advantage in targetting spectral acquisition to a specific leaf surface (judged by correlation coefficients and root mean square error for the cross-validation). Spectra were acquired daily for 14 days. Thereafter, the process was conducted after 16, 18, 22 and 30 days (30 days coinciding with day 89 from the commencement of the irrigation treatments). Fresh seedlings were sampled on each occasion. After acquiring the NIR spectra and physiological trait measurements, the seedlings were removed from the trial.

Measurement of relative water content and water potential

The same leaf pairs used for collection of NIR spectra (described above), were used for measurements of relative water content and midday leaf water potential. Spectra from each of the leaves of each selected seedling were acquired shortly prior to their removal for physiological measurements, with the aim of minimising time between spectral acquisition and physiological assessments. All this work was done close to midday.

One leaf out of the pair was removed from the seedling and immediately had its water potential measured using an a water potential meter. This unit used a pressure chamber of the 'Schulander' type to quickly and efficiently measure water potential. The representative leaf was inserted into a small chamber which was then pressurised. Water potential (in MPa) was then obtained directly from the reading provided by the instrument.

The other leaf of the pair was also removed from the seedling for determining relative water content (Barrs & Weatherley 1962). Fresh weight (W_f) of the leaf was measured immediately after removal. The leaf was then cut it into halves along its main vein and these halves were immersed in distilled water in a labelled petri dish at 20 °C, and under florescent light. Separate petri dishes were used for each leaf/seedling sample. After 4 hours, the leaf halves were removed from the water, excess water removed from their surfaces using filter paper, and they were then weighed to obtain saturated weight (W_s). The two halves were then inserted into labelled paper envelopes and placed in an oven at 60 °C for 48 hours. The leaf halves were then weighed again to obtain leaf dry weight (W_d). The relative water content (RWC) value for each sample leaf/seedling was then calculated as follows:

$$RWC = (W_f - W_d) / (W_s - W_d) \times 100\%$$

Data statistics and analyses

Sample spectra were first screened for outliers using two methods. All the NIR spectra were plotted to visually check for any spectral profiles that appeared abnormal by differing markedly from the mean spectrum. An initial principal component analysis was conducted using The Unscrambler version 9.7 software to check for any samples having spectrum with unusually high residual variance. One sample was subsequently eliminated from the data as an outlier. The remaining 215 samples—spectra matched with relative water content and water potential—were randomly divided into two sets, one for calibration with 185 samples, and one for validation set containing 30 samples.

To normalise variances of the traits of interest (i.e. relative water content and water potential) the trait measurements were standardised by subtracting the mean and dividing by the standard deviation of the trait prior to further analyses. Multivariate analyses were then performed using The Unscrambler following methodology described by Warburton et al. (2014). Both raw spectra and pretreated spectra first or second derivative, three-point smoothing, second-order polynomial (Savitzky & Golay 1964), were used in the development of partial least squares regression calibrations. Development of the calibration for both water potential and relative water content was performed using full cross-validation. Optimum rank of the calibration was chosen to be that at which the first local minimum in residual variance occurred.

The performance of the calibration models developed for predicting water potential and relative water content was evaluated using the validation set (30 samples). For each of the 30 samples the estimated water potential and relative water content predicted from their NIR spectra (using the calibrations developed from the 185-sample set) were compared with the actual measured values. The root mean square errors (RMSE) and root mean square errors of prediction (RMSEP) of the average difference between predicted and measured response values, at the calibration and validation stage respectively, were also estimated.

RESULTS

Means, coefficients of variation (CV) and ranges of relative water content and water potential from both the calibration and validation sets of *E. camaldulensis* seedlings are presented in Table 1. Despite many of the seedlings being irrigated with 50% or less of the potential water, loss from their pots through evapotranspiration, relative water content never dropped below 55% in any of the seedlings and the minimum water potential recorded was -1.84 MPa.

Allocation of seedlings between calibration and validation sets was done randomly, albeit with more seedlings allocated to the calibration set (185 seedlings) than to the validation set (30 seedlings). Even so, the means and coefficients of variation of relative water content and water potential were similar between these sets, though the ranges of both relative water content and water potential were somewhat wider in the calibration set, likely due to the much greater number of seedlings in the set. The wide ranges of relative water content and water potential observed, especially in those of the calibration set, was desirable for successfully developing a robust NIR calibration model for this species.

Figure 1 shows the loadings plot of the first principal component (PC1), estimated from the principal component analysis of the raw NIR spectrum from *E. camaldulensis* leaves. The greatest fluctuations in both raw spectra and principal component loadings were in the region between 1860 and 1960 nm. A second derivate transformation enhanced characteristic peaks

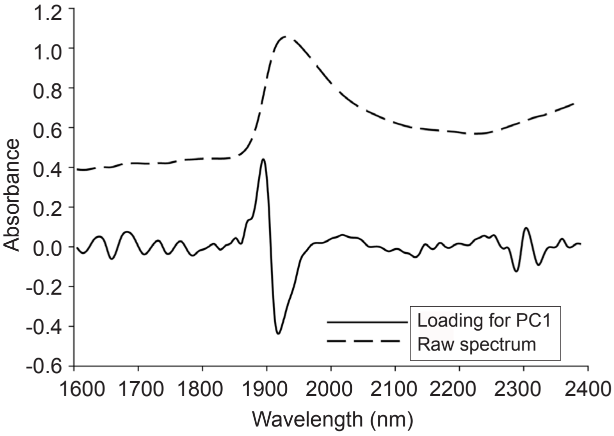

Figure 1 Plot of loading weights for first principal component (PC1) and the raw spectrum from principal component analysis (scores of factors) of all samples

Table 1 Descriptive statistics of relative water content (RWC) and leaf water potential (Ψleaf) from the calibration and test set validation of *Eucalyptus camaldulensis* seedlings

Set	Number of seedlings	Trait	Mean	CV	Range
Calibration	185	RWC	80.2%	7.9%	55.8 – 94.1%
		Ψleaf	-1.10 MPa	23.9%	-1.84 – -0.53 MPa
Validation	30	RWC	81.0%	6.9%	69.7 – 90.9%
		Ψleaf	-1.11 MPa	22.8%	-1.59 – -0.69 MPa

CV = coefficient of variation

and hence helped reveal more information behind the NIR spectrum associated with variations in leaf properties.

Figure 2 shows plots of loadings on the first principal components used in the subsequent partial least squares regressions for relative water content and water potential. Results of partial least squares between the NIR spectra and the two physiological variables (relative water content and water potential) are given in Table 2. Figures 3 and 4 show plots for relative water content and water potential respectively of the predicted values based on the models derived from the partial least squares analyses versus the measured values. A stronger coefficient of determination was obtained for the water potential calibration set, R^2_C = 0.879 using 10 factors, than was obtained for relative water content calibration set, R^2_C = 0.828 using 12 factors.

The percentages of variance explained for X and Y by the principal components involved in the calibration model for estimating relative water content and those for leaf water potential are presented in Table 3. For water potential, 79% of the variance in X (NIR spectra) accounted for 47% of the variation in Y (leaf water potential) using the first three factors, whilst for relative water content 79% of the variation in X explained 36% of the variation in Y using the first three factors.

If judged by their coefficients of determination (R^2) and root mean square errors from the validation set (0.83 and 2.45 for leaf relative water content and then 0.89 and 0.08 for water potential respectively, Table 2), then the calibrations obtained appeared relatively robust. When these calibrations were then used to predict relative water content and water potential values on a

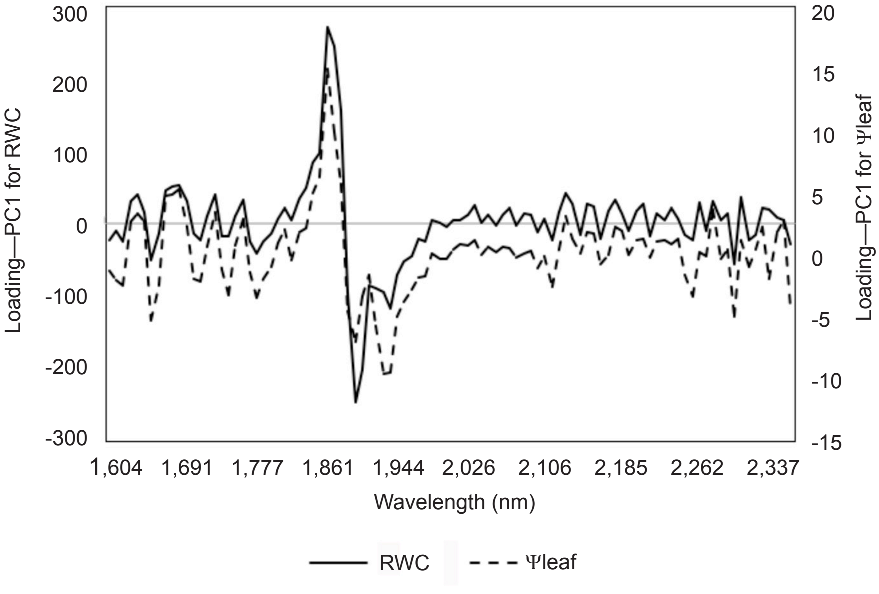

Figure 2 Plot of loading weights for the first principal component (PC1) used in partial least squares regressions of relative water content (RCW) and leaf water potential (Ψleaf) of *Eucalyptus camaldulensis* leaves

Table 2 Calibration statistics for calibration and validation test set for relative water content (RCW) and leaf water potential (Ψleaf) of the *Eucalytus camaldulensis* seedlings

Trait	PC	Calibration		Validation test set			
		R^2_C	RMSEC	R^2_P	RMSEP	Slope	Bias
RCW	12	0.83	2.45	0.40	6.64	0.70	0.02
Ψleaf	10	0.88	0.08	0.80	0.14	0.79	-0.0007

PC = number of factors (principal components) used in the model, R^2_C = coefficient of determination for the calibration model, RMSEC = root mean square error of calibration, R^2_P = coefficient of determination for the test set prediction, RMSEP = root mean square error of prediction

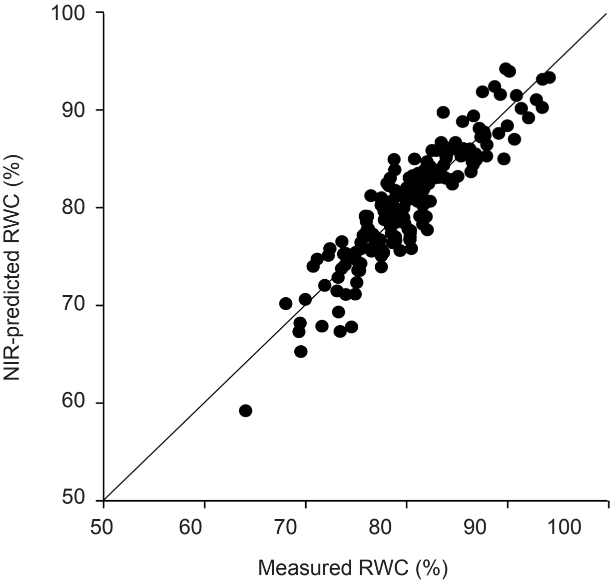

Figure 3 Near infrared (NIR)-predicted versus measured calibration plot for relative water content (RCW) in leaves of *Eucalyptus camaldulensis*; $R^2_C = 0.83$, root-mean-square error of calibration = 2.45

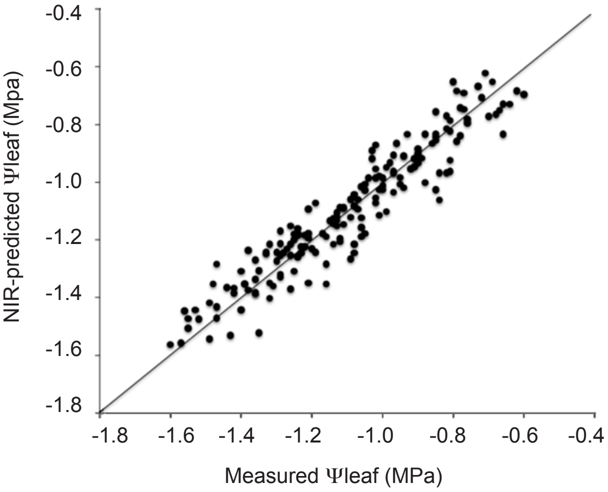

Figure 4 Near infrared (NIR)-predicted versus measured calibration plot for leaf water potential (Ψleaf); $R^2_C = 0.89$, root-mean-square error of calibration = 0.08

separate validation test set (30 seedlings), the coefficient of determination (R^2_P) and root mean square error of prediction for water potential were 0.78 and 0.14 respectively, indicating relatively accurate prediction. However, for relative water content, the values were 0.41 and 6.64 respectively, indicating relatively poor accuracy of prediction.

In this study, the seedlings used to develop the NIR calibration model were grown under four different irrigation treatments, with NIR scans and physiological measures being taken at 19 different times over a period of 30 days. Consequently, both sets of seedlings represented a wide range of water stress conditions, and this should have provided some confidence and robustness in the calibrations developed.

DISCUSSION

The greatest variation in the NIR spectral reflectance from the *E. camaldulensis* leaves examined in this study seemed to occur in the range of wavelengths from 1860 to 2030 nm (Figure 1). This spectral region has been shown by previous studies to correspond to a dominant peak for water (Shenk et al. 2001, Schwanninger et al. 2011). However, it is difficult to determine if a specific spectral region is correlated with just a single, specific chemical compound, since any one narrow characteristic peak of NIR spectra may in fact represent multiple compounds (Foley et al. 1998).

Some studies suggested that the spectral regions between both 1400–1440 and 1900–1950 nm have particularly strong NIR reflectance or absorbance bands associated with water, and these wavelengths have often been used to analyse water content in plants (Schwanninger et al. 2011, Workman & Weyer 2012). However, of these regions only the latter (1900–1950 nm) was measured in this study, the former being outside of the spectral range of the NIR spectrometer used.

The abundance of variation within the two traits of interest in the samples examined for NIR spectra analyses affected the precision and robustness of the NIR-calibrated models; broader variation being desirable for developing robust models. Generally, such a requirement can be satisfied by collecting samples from a wide range of environments and from a wide genetic base (Poke et al. 2006, Stackpole et al. 2011). This was done in this current study on *E. camaldulensis*, albeit without retaining genetic origins of individual seedlings through to the evaluation stages. That there was indeed abundant variation in the traits of interest in the sample used for analyses in this current study was confirmed by the parameters provided in Table 1. Had the sample size (number of seedlings) been increased and the genetic origins or pedigrees maintained (down to the family level), this might have been advantageous to improving the calibrations.

Table 3 Percentage of variance explained for X and Y by principal components involved in the calibration models for estimating relative water content and leaf water potential of *Eucalyptus camaldulensis* seedling leaves using NIR spectra

Principal component number	Relative water content		Water potential	
	% variation for X explained by principal component	% variation for Y explained by principal component	% variation for X explained by principal component	% variation for Y explained by principal component
PC1	38	17	33	19
PC2	13	15	13	25
PC3	28	4	33	3
PC4	2	15	2	18
PC5	3	5	3	4
PC6	1	7	1	6
PC7	2	5	1	5
PC8	1	5	2	2
PC9	1	4	1	3
PC10	1	2	1	1
PC11	1	1	n.a.	n.a.
PC12	0	2	n.a.	n.a.

n.a. = calibration model for water potential included 10 principal components

Strong heritability of many traits in *Eucalyptus*, or at least *E. globulus*, may somewhat reduce the abundance of the sample variation. Therefore, estimating the heritabilities for traits of interest before choosing the samples for evaluation may assist in providing phenotypically diverse samples for at least the calibration set (Stackpole et al. 2011).

The NIR-calibrated model developed in this study for midday water potential proved more accurate (high R^2) in both the calibration and validation sets than did that for relative water content. Different performance of the two NIR-calibrated models might be due in part to the very different methodologies involved in measuring water potential and relative water content and that there was slightly longer time period between acquiring the NIR scan of a leaf and commencing the relative water content determination. Even over a short time period, particularly once a leaf has been removed from the stem, rapid and large change in the physiological condition can occur and it is highly possible that the extent of the change in relative water content and water potential in leaves over different time periods may be significant.

In a recent study conducted using NIR spectra obtained from leaf surfaces of *E. grandis* seedlings to develop calibrations for leaf physiological traits, Warburton et al. (2014) reported stronger coefficients of determination for relative water content than leaf water potential (R^2 = 0.85 and 0.74 respectively for the validation test set). These values contrasted the current study on *E. camaldulensis* which obtained stronger coefficients of determination for leaf water potential than for relative water content. Part of the reason for the contrasting results between the studies may well lie in the very different leaf properties of the two species. Leaves of *E. camaldulensis* is somewhat duller, waxier and with thicker cuticles than those of *E. grandis* which has softer, glossier leaves. The absence or presence of a thick cuticle or waxes, and various other leaf properties can all influence reflectance and absorbance of spectra by leaves (Bei et al. 2011).

CONCLUSIONS

This study demonstrated the potential of using NIR spectroscopy to rapidly and non-destructively predict leaf water potential in *E. camaldulensis.* However, the results also indicated that more work will be required before the same can be achieved for predicting leaf relative water content with acceptable accuracy. Further work will also be required to evaluate if the calibrations developed for *E. camaldulensis* can be applied to other *Eucalyptus* species and to develop a model for prediction of water status traits in populations of all *Eucalyptus* species and possibly also those of other plantation tree genera.

The ability to use NIR non-destructively in studies of leaf water status will facilitate drought response and recovery research, and could enable measurements to be made non-destructively in field/plantation settings. Development of such capabilities would be highly beneficial for tree breeders endeavouring to select and breed genotypes for environments where water availability can be severely limiting.

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