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Hyperspectral reflectance as a tool to measure biochemical and physiological traits in wheat

Predicting biochemical and physiological traits in wheat

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ABSTRACT
Improving photosynthesis to raise wheat yield potential has emerged as a major target for wheat physiologists. Photosynthesis related traits, such as nitrogen per unit leaf area ($N_{\text{area}}$) and leaf dry mass per area (LMA) require laborious destructive laboratory based methods, while physiological traits underpinning photosynthetic capacity, such as maximum Rubisco activity ($V_{\text{cmax}25}$) and electron transport rate ($J$), require time-consuming gas exchange measurements. The aim of this study was to assess whether hyperspectral reflectance (350 to 2500 nm) can be used to rapidly estimate these traits on intact wheat leaves. Predictive models were constructed using gas exchange and hyperspectral reflectance data from 76 genotypes grown in glasshouses with different nitrogen levels and/or in the field under yield potential conditions. Models were developed using half of the observed data with the remainder used for validation, yielding correlation coefficients ($R^2$ values) of 0.62 for $V_{\text{cmax}25}$, 0.7 for $J$, 0.81 for SPAD, 0.89 for LMA and 0.93 for $N_{\text{area}}$, with bias <0.7%.

The models were tested on elite lines and landraces that had not been used to create the models. The bias varied between -2.3% and -5.5% while relative error of prediction was similar for SPAD but slightly greater for LMA and $N_{\text{area}}$.

Key words: Electron transport rate, hyperspectral reflectance, leaf dry mass per area, leaf nitrogen, partial least squares, photosynthesis, Rubisco, Triticum aestivum, velocity of carboxylation.
INTRODUCTION

Global population is predicted to reach 9.7 billion by 2050 (U.N., 2015). To satisfy projected demand for cereal grain, wheat yields need to increase at rates far exceeding the current annual genetic gains being made in most parts of the world by plant breeders (Reynolds et al., 2012). Further improvements in yield require increases in biomass, derived from improvements in radiation use efficiency and photosynthetic traits (Parry et al., 2011; Reynolds et al., 2012). Despite its importance, selection based on physiological and biochemical characteristics of wheat genotypes in a breeding program is uncommon due to cost and the time required for testing at a breeding scale. The development of tools that improve speed and accuracy of estimating biomass and photosynthesis-related traits would allow screening of a large number of lines, making these traits more amenable to incorporation into breeding programs. This would also facilitate identification of molecular markers and candidate genes underpinning genetic variation for the traits of interest. Spectral reflectance is associated with specific plant characteristics and has been proposed as a fast and non-destructive technique that can be efficiently used in breeding programs where thousands of individuals must be screened every year (Babar et al., 2006).

Prediction of photosynthesis-related traits through simple leaf reflectance parameters is well established. Reflectance in the visible/near infrared part of the electromagnetic spectrum has been related to xanthophylls, chlorophylls, and water in plants, and the red edge in the derivative of reflectance is commonly related to photosynthesis (Peñuelas and Filella, 1998). One of the first and most widely used optical instruments is the SPAD chlorophyll meter. This measures transmittance of red (560 nm) versus infrared (940 nm) light to estimate leaf chlorophyll content (Benedict and Swidler, 1961; Inada, 1963; Mullan and Mullan, 2012). Numerous indices based on wavelengths in the visible and infrared part of the electromagnetic spectrum have been used in remote sensing to predict vegetation biomass, biochemical leaf components and some physiological traits. For example, normalized difference vegetative index (NDVI) is used to monitor vegetation using red, infrared and near-infrared wavelengths to measure relative greenness, foliage development, senescence, biomass and chlorophyll content (Tucker, 1979; Goward et al., 1985; Gamon et al., 1995; Cabrera-Bosquet et al., 2011; Lopes and Reynolds, 2012; Pinto
et al., 2016). The water index (WI) is used to infer water content from reflectance ratios between 900 and 970 nm (Peñuelas et al., 1997) while the photochemical reflectance index at 531 and 570 nm (PRI) has been used to estimate radiation-use efficiency and photoprotective pigment pools in leaves (Gamon et al., 1992; Peñuelas et al., 2011).

The infrared (IR) region is commonly divided into three regions: near infrared (NIR, 770-1300), short wave infrared 1 (SWIR1) region (1300-1900 nm), and short wave infrared 2 (SWIR2) region (1900-2500 nm). Research in the IR part of the spectrum has increased because hyperspectral cameras and field spectroradiometers are increasingly able to accurately measure the full spectrum (i.e. 350-2500 nm) and because the incorporation of information from the entire visible to SWIR2 region has proven useful for a range of plant traits (e.g. Singh et al., 2015; Yang et al., 2016). IR spectra measured from leaves have been correlated with photosynthetic parameters (maximum Rubisco activity, \( V_{cmax} \) and electron transport rate, \( J \)) (Serbin et al., 2012; Ainsworth et al., 2014), and have been used to predict carbon, nitrogen and phosphorus content of leaves (Gillon et al., 1999). Successful predictions of photosynthetic parameters have been obtained for tropical trees, aspen, cotton, soybean and maize (Doughty et al., 2011; Serbin et al., 2012; Ainsworth et al., 2014; Yendrek et al., 2017), and nitrogen content and LMA in wheat (Ecarnot et al., 2013). In wheat at the canopy level, predictions from hyperspectral reflectance for biomass, nitrogen and water content have been demonstrated (Hansen and Schjoerring, 2003; Pimstein et al., 2007; Yao et al., 2015). These examples show the potential of using hyperspectral reflectance to screen wheat for photosynthetic parameters (Garriga et al., 2017).

The main objective of this study was to develop statistical models linking leaf-level hyperspectral reflectance to photosynthetic traits, thereby establishing a high throughput alternative to the traditional time-consuming methods. Leaf reflectance spectra are correlated with photosynthetic traits derived from the response of \( CO_2 \) assimilation to \( CO_2 \) concentration using the model of Farquhar, von Caemmerer and Berry (1980) considering the new parameters for wheat (Silva-Pérez et al., 2017). The method is validated for \( V_{cmax} \), (correlated with Rubisco activity), \( J \) (electron transport rate) and with LMA, \( N_{area} \) and SPAD (a surrogate for chlorophyll content).
Examples are given where the derived models are used to predict SPAD, LMA and \( N_{\text{area}} \) in two previously unseen sets of elite and landrace wheat genotypes.

**MATERIALS AND METHODS**

**Plant Material**

Six sets of diverse wheat (*Triticum aestivum*, *T. turgidum*) and triticale germplasm were used in these experiments as follows: (1) Early Vigour (EV), 16 wheat genotypes from CSIRO in Australia. Most of the genotypes have a larger embryo, fast leaf area development and low leaf mass per unit area. (2) A subset of the Best and Unreleased Yield Potential (BYP), 21 wheat genotypes and 9 triticale genotypes with high yield in Australia. (3) CIMMYT Core Germplasm Subset II (C), 30 wheat genotypes selected at CIMMYT (International Maize and Wheat Improvement Center) for high yield (González-Navarro *et al.*, 2015). (4) Candidates of C (CC), 216 elite wheat genotypes plus seven wheat genotypes from C, in total giving 223 wheat genotypes. (5) Wheat landraces (L) obtained from CIMMYT’s gene bank; 230 wheat landraces plus five elite wheat genotypes including two from CC, giving 235 wheat genotypes in total. (6) A subset of L (LS), 23 genotypes with similar phenology. An additional letter added to each acronym indicates whether the measurements were made before anthesis (B) or at anthesis (A).

**Experimental conditions**

The Zadoks scale was used to describe the growth stages (GS) of wheat (Zadoks *et al.*, 1974). The first day after emergence (DAE) is considered at GS10, when at least 50% of the first leaves emerging through coleoptile are visible. Five experiments were conducted: **Aus1**, **Aus2**, **Aus3**, **Mex1**, **Mex2** (Table1):

The first glasshouse experiment, **Aus1**, was set up at CSIRO Black Mountain, Canberra, Australia (-35.271875, 149.113982). Two seeds of EVA set were sown in cylindrical pots of 1.06 L (15 × 5cm) with 75:25 loam:vermiculite containing basal fertilizer, and one plant per pot was kept for the experiment. Plant emergence was on 8\(^{\text{th}}\) April, 2012, artificial light was used in June to extend the photoperiod to 16 h and temperature was controlled to 25/15 °C (day/night). **Aus1** was designed to achieve a range in leaf colour with nitrogen deficiency in one treatment (-N) and high fertilizer in the other treatment (+N), and the experiment was organized in a
randomized block design, three blocks representing each repetition for +N and other three blocks -N. Extra fertilizer Thrive (~300 mL per pot of 1.77g L⁻¹N:27%, P:5.5%, K:9%) was applied each week for +N treatment until 83 DAE. A severe low nitrogen treatment was obtained irrigating the pots with water without fertilizer 1.5 month before measurements. The flag leaf was measured at the end of booting and during anthesis (GS58-69) from 73 to 83 DAE.

The second glasshouse experiment, Aus2, was carried out at CSIRO Black Mountain, Canberra, Australia. Three seeds of BYPB set were sown in pots of 5 L with 75:25 loam:vermiculite soil mix containing basal fertilizer, and two plants per pot were kept for the experiment. Plant emergence was on 17 October 2012 and temperature was controlled to 25/15 °C (day/night). Aus2 was organized in a randomized block design, two blocks representing each repetition for the high nitrogen treatment (+N) and one block for the low nitrogen treatment (-N). For the +N treatments extra fertilizer Aquasol (~300 mL per pot of 1.77g L⁻¹N:23%, P:4%, K:18%) was applied every three days from 41 to 56 DAE. Treatment -N was obtained irrigating the plants with water without fertilizer 10 days before measurements. Treatment -N was applied over a shorter duration than Aus1, resulting in smaller differences in leaf nitrogen content per unit leaf area and photosynthetic parameters. The flag leaf was measured before anthesis (GS49-57) from 48 to 56 DAE.

Experiment Aus3 was carried out in the field at CSIRO Experimental Station at Ginninderra, Australia (-35.199837, 149.090898). The emergence of plants was on October 4th, 2013. From 1 to 75 DAE the average maximum from daily temperature (Fig. S1) was 22.4 °C and the minimum 7.7 °C, in total 142 mm of rain and an accumulative thermal time of 1,126.8 °C d (base temperature 0°C). Average solar radiation 24 MJ m⁻² (Fig. S1). Due to late sowing and long days (~11 h) the wheat cycle was short. CA and EVA subsets of wheat genotypes were sown in the same experimental design of two randomized blocks. Each block was subdivided into 30 plots (5 × 6). Next to this experimental design, another experimental design of two randomized blocks for the BYPB collection was sown. In this case, each block was subdivided into 42 plots (7 × 6). Each plot for both experimental designs was 5 m × 1.8 m. It contained a single genotype sown in 10 rows, 18 cm apart, and
approximately 200 plants m$^{-2}$. Plots were fertilized and irrigated optimally in all conditions. For the BYPB subset of wheat genotypes, the flag leaf was measured before anthesis (GS40-55, 46-54 DAE) where the maximum and minimum temperatures were 28.3 °C and 5.4 °C, respectively. The maximum and minimum temperatures during measurement of EVA (GS69, 62-67 DAE) and CA (GS56-69, 60-67 DAE) were 32.2 °C and 4.3 °C, respectively. Measurements and sampling were done twice in two plots, resulting in 4 repetitions for 4 to 5 genotypes per day that were at similar plant stage. Due to the close phenology among the lines studied, the number of genotypes measured was reduced: 2 wheat genotypes from EVA, 20 wheat genotypes and 6 triticale genotypes from BYPB, and 22 wheat genotypes from CA.

Experiment Mex1 was carried out in the field at Centro Experimental Norman E. Borlaug (CENEB) research station, located in the Yaqui Valley, Sonora, Mexico (27.370837, -109.930362) for a winter-spring cycle. Plant emergence was on 2$^{nd}$, December, 2012. From the 1 to 138 DAE, the average maximum temperature from daily temperature (Fig. S1) was 26 °C and the minimum 8.3 °C, in total 15.38 mm of rain and an accumulative thermal time of 2,364.6 °C. Average solar radiation 17 MJ m$^{-2}$ (Fig. S1). Plants were organised in a randomised 5 × 6 lattice experimental design with three repetitions. Each repetition (10 × 3 plots) enclosed two subdivisions of 5 × 3 plots. Each plot (2.4 m × 8.5 m) contained a single genotype sown in 6 rows, two beds in the middle with two rows each and two beds in the edges with one row of the same genotype, the second row in the edges corresponded to the next genotype or a filling genotype to avoid border effect. Beds followed the system 56-24, where 56 cm is the furrow width and 24 cm is the raised bed width. Plants were grown under optimal management in the field. There were in total five auxiliary irrigations, with a total of 500 mm of water applied. First fertilization was at soil preparation with 50 kg ha$^{-1}$ of N and 50 kg ha$^{-1}$ of P and a second fertilization in the first irrigation of 150 kg ha$^{-1}$ of N. For the CB subset of wheat genotypes, the flag leaf was measured before anthesis (GS49-57, 67 to 82 DAE), with maximum and minimum temperatures of 29.7 °C and 1.5 °C, respectively. For the CA subset, flag leaves were measured at anthesis (GS65+7, 88 to 103 DAE), with maximum and minimum temperatures of 32.1 °C and 2.5 °C, respectively. Measurements and sampling were done in one plant per plot; 3 to 6 genotypes per
day were measured at similar plant stage with 3 repetitions.

Field experiment Mex2 was used to test the reflectance method developed in this study in a larger, diverse group of wheat genotypes. CC and L genotypes were sown at the same time and near the plots from the Mex1 experiment at CENEB during the same season with same sowing date, same plant emergence date, similar weather conditions apply (Fig. S1) and the same crop management. Plots in both sets of wheat genotypes were 2 m long × 1.6 m, each one contained two beds arranged in the 56-24 system. CC plants were arranged in the field in 20 × 22 plots plus 6 plots in the 23rd row of plots to give 446 plots in total, the whole experiment comprised two randomized blocks. L plants were sown in a band of 5 × 54 plots. From these 270 plots, 230 plots contained single landrace wheat genotypes and 40 plots contained elite wheats (checks), placed after every ten landrace plots. The measurements were done in two main steps: 1) Survey: CC and L flag leaves were measured for reflectance and SPAD on all plots including repetitions and checks. CC (n=446) plants were measured from 101 to 103 DAE, they were 15 days after anthesis on average. L plants (n=270) were measured from 110 to 111 DAE, genotypes varied from 1 to 36 days after anthesis (Fig. S5). 2) Second measurement: After the survey, 23 L genotypes that were 5 - 10 days after anthesis were selected from the survey (Fig. S5) and measured a second time (LS) for reflectance, SPAD and sampled to calculate leaf mass area (LMA) and leaf nitrogen content per unit leaf area (N\text{area}).

Measured traits

Gas exchange was measured using a LICOR LI-6400XT infrared gas analyser (LI-COR Inc., Lincoln, NE, USA); the 6 cm² rectangular head was used for experiments Aus1, Aus2 and Aus3, and the 2 cm² circular fluorescence head (Li-6400-40; LI-COR Inc.) for Mex1 experiments. The flow rate into the leaf CO₂ chamber of the Li-Cor was set at 500 μmol s⁻¹ for the 6 cm² head and 350 μmol s⁻¹ for the 2 cm² head, irradiance was held at 1800 μmol quanta m⁻² s⁻¹, and block temperature at 25 °C. Gas exchange was used to measure the rate of CO₂ assimilation (A) and stomatal conductance (gₛ) at 400 inlet μmol CO₂ mol⁻¹ initially followed by a CO₂ response curves (inlet CO₂ values used are shown in Table S1). The maximum Rubisco activity normalised to 25 °C, V\text{cmax25}, and electron transport rate, J, were calculated
using the leaf biochemical model of photosynthesis (Farquhar et al., 1980) with kinetic constants derived for wheat (Silva-Pérez et al., 2017).

Flag leaves were measured with a SPAD-502 chlorophyll meter (Minolta Camera Co., Ltd, Japan) to provide a non-destructive surrogate for chlorophyll content (Mullan and Mullan, 2012). In all experiments, 3 SPAD readings taken from the same region of the leaf used for leaf reflectance and gas exchange measurements were averaged per leaf.

Following gas exchange experiments in Aus1, Aus2 and Aus3, leaf material was sampled three centimetres up and down the leaf from where the chamber was clipped on in order to determine leaf mass per unit area and nitrogen concentration. Area of the leaf samples was calculated from a digital photo using the program ImageJ 1.47v. Samples were then dried for 48h at 70°C to achieve constant mass and weighed on an analytical balance (Mettler Toledo, AT201, 0.01 mg) to obtain leaf mass area (LMA, g m⁻²). Leaf nitrogen concentration, N_mass (N mg g⁻¹) and phosphorus concentration P_mass (P mg g⁻¹) were determined on the same samples by flow injection analysis (QuikChem® Method, Lachat Instruments, CO, USA) after Kjeldahl digestion of leaves. For Mex1 and LS-Mex2 experiments, a complete flag leaf was measured using a leaf area meter (LI3050A/4; LICOR, Lincoln, NE), followed by drying for 48h at 70°C and weighing on a precision balance (Ohaus Adventurer, AR1530, 0.001g) to obtain leaf mass area (LMA, g m⁻²). Nitrogen concentration, N_mass (N mg g⁻¹) was determined at CIMMYT Batan, Mexico with the Technicon AutoAnalyzer II (Galicia et al., 2008). N_mass or P_mass and LMA were used to calculate nitrogen content per unit leaf area N_area (N g m⁻²) and phosphorous content per unit leaf area P_area (P g m⁻²).

**Reflectance measurements**

Reflectance spectra were measured with a FieldSpec®3 (Analytical Spectral Devices, Boulder, CO, USA) full range spectroradiometer (350-2500 nm) coupled via the fibre optic cable to a leaf clip with an internal calibrated light source and with two panels, a white panel used for instrument calibration and a black panel used for measurements (Analytical Spectral Devices, Boulder, CO, USA). The calibration (i.e. white reference) of 100 reflectance spectra took 20 s and the leaf measurement took maximum 30 s in Aus1 experiment. At this stage, reflectance was measured using
two pieces of leaf measured in the horizontal position (Fig. S2.A). The technique was improved in **Aus2, Aus3, Mex1** and **Mex2** experiments, where the calibration of 30 reflectance spectra took 6 s and the leaf measurement took 9 s, each leaf was placed vertically, which helped to speed up the measurements in the field (Fig. S2.B). In these experiments a mask was used to reduce the leaf-clip aperture to an elliptic area of 1.264 cm² (1.15 × 1.4 cm) suitable for wheat leaves, a black circular gasket of 2.2 cm inner diameter and 3 mm thick was pasted to the mask to avoid leaf damage and to eliminate potential entry of external light through the edges (Fig. S2.C). In experiments **Aus1, Mex1** and **Mex2**, one reflectance measurement was made per leaf lamina, two in **Aus2**, and three in **Aus3**, which were averaged. The leaf lamina repetitions are independent from the experimental design repetitions.

**Analysis of leaf reflectance spectra**

Leaf spectra required pre-treatment to correct for the “jump” observed in apparent reflectance when changing between the detectors. First, two different jump corrections were applied to the reflectance measurements because two different ASD FieldSpec®3 spectroradiometers were used, one in Australia and the other in Mexico. Reflectance measured with the FieldSpec3 in Australia was corrected at 1000 nm and 1800 nm. Reflectance measured with the FieldSpec3 in Mexico was corrected at 1000 nm and 1830 nm using the software Spectral Analysis and Management System (SAMS®), version 3.2. Spectra with reflectance lower than 0.35 and higher than 0.6 at 800 nm were removed because an earlier analysis had shown these to be outliers. Finally only from 400 to 2400 nm from the spectrum was used in the analysis.

Analysis of the reflectance data was performed using the ‘pls’ package ‘Principal Component and Partial Least Squares Regression in R’ (Mevik and Wehrens, 2007) under R software version 2.15.0. One or two repetitions from experiment **Aus1, Aus2, Aus3** and **Mex1** were used as training data (about 55% of the total observed data) to ensure that the complete set of genotypes are present in both training and test data (Table S2). The remaining repetitions from experiment **Aus1, Aus2, Aus3** and **Mex1** were used only as test data (about 45% of the observed data) to validate the partial least square regression (PLSR) models. The number of components used in the regression model fitted to the reflectance data was based in the smaller root
mean square error of the cross validation (RMSEP-CV) and the smaller predicted residual sum of squares (PRESS) from the training data. PLSR generates loadings and scores that are used to generate a group of regression coefficients for each wavelength and an intercept, which we call PLSR model. The PLSR model is different for each trait (Fig. S3). An example of the reflectance measurements, loadings and regression coefficients for 18 components obtained for \( \nu_{cmax25} \) is shown in Fig. 1.

Evaluation of the model accuracy included the coefficient of determination \( (R^2) \), the model bias:

\[
Bias \ (\%) = 100 \times \frac{\bar{\hat{y}} - \bar{y}}{\bar{y}}
\]

(1)

to represent percentage of the difference between the mean of the predicted trait, \( \bar{\hat{y}} \), and the mean of the observed trait \( \bar{y} \),

and the Relative Error of Prediction (REP) (Nguyen and Lee, 2006):

\[
REP \ (\%) = 100 \times \left[ \frac{1}{n} \sum_{i=1}^{n} (y_i - \hat{y}_i)^2 \right]^{0.5} / \bar{y}
\]

(2)

to represent the percentage of the root mean square error in prediction, where \( y_i \) and \( \hat{y}_i \) are observed and predicted traits, \( n \) is the number of sample in data set and \( \bar{y} \) is the mean of the observed values of traits.

**Applying the PLSR models**

One objective of this study was to assess whether leaf-level hyperspectral reflectance could be used as a high throughput alternative to traditional and time-consuming measurements of destructive analyses for biomass-related and photosynthetic traits. Experiment (Mex2) included 458 elite wheat genotypes (CC-Mex2) and landraces (L-Mex2) (Table 1) that were independent from the genotypes used to train and validate the models. They were surveyed with hyperspectral leaf reflectance and SPAD. At the time the leaf reflectance survey of wheat landraces was made, their phenological development ranged from seven days before to 36 days after anthesis (Fig. S3). Consequently, 21 wheat landraces and two elite wheats (checks) between six and nine days after anthesis were selected for the LS-Mex2 experiment, where hyperspectral leaf reflectance was measured and leaves
were sampled to obtain LMA and N\textsubscript{area}.

RESULTS

Predictions and validation of traits

Predictions for N\textsubscript{area}, LMA and SPAD had higher coefficients of determination than for the photosynthetic parameters and observations followed the 1:1 line (Fig. 2, bias <0.7% Table 2). For these traits, the residuals were smaller and showed no underlying trends. N\textsubscript{mass} had a smaller coefficient of determination than N\textsubscript{area} ($R^2=0.7$ vs $R^2=0.93$) (Table 2).

Two predictions are shown for the Rubisco related trait $V_{cmax}$: 1) $V_{cmax}$ without leaf temperature correction and 2) $V_{cmax25}$ corrected to a common leaf temperature of 25 °C using \textit{in vivo} Rubisco kinetics derived for wheat (Silva-Pérez \textit{et al.}, 2017). Both predictions fall approximately on the 1:1 line (Fig. 3, bias <0.2%). The residuals between observed data and predictions were larger for $V_{cmax}$ than $V_{cmax25}$.

In the case of $J$, predictions fell about the 1:1 line with the coefficient of determination ($R^2=0.71$) slightly less than for $V_{cmax}$ ($R^2=0.74$, Fig. 2). The trend of $J$ predictions and residuals are similar to $V_{cmax25}$.

When Kjeldahl digestion was used to determine leaf nitrogen, we also obtained a measure of phosphorus. Predictions of leaf phosphorus from hyperspectral reflectance were not as good as for nitrogen ($P_{mass} R^2=0.65$, $P_{area} R^2=0.42$) (Table 2).

Predicting $V_{cmax25}/N_{area}$

Given the fact that CO\textsubscript{2} assimilation rate, $A$, and stomatal conductance, $g_s$, are variable for a given leaf and depend on environmental conditions, it was not surprising that their prediction was generally low ($A R^2=0.49$, $g_s R^2=0.34$, Table 2). Instead, we targeted underlying photosynthetic capacity normalised per unit leaf nitrogen, $V_{cmax25}/N_{area}$. For this trait, which represents photosynthetic efficiency (Rubisco capacity per unit leaf N), the model predictions fell about the 1:1 line ($R^2=0.49$, bias 1.9%) (Fig. 4). Interestingly, the coefficient of determination for $V_{cmax25}/N_{area}$ predicted as a ratio, was greater than when the trait was calculated from the ratio of values of $V_{cmax25}$ and N\textsubscript{area} predicted separately ($R^2=0.13$).
In general, the residuals showed no underlying trends when plotted against the predicted data (Fig. 2, 3 and 4). However, there was a positive trend within each experimental group when residuals were plotted against observed data (Fig. S4).

**Predicting traits for novel wheat genotypes that were not used for PLSR model derivation**

To assess the use of hyperspectral reflectance as a high throughput tool in the field, 458 elite wheat genotypes and landraces (Mex2) were surveyed. The predicted values of SPAD fell about the 1:1 line and the relative error of prediction for SPAD compared favourably to that observed for the validation data (CC-Mex2 7.4% and L-Mex2 6.6%, Table 3; cf 6.78%, Table 2). The distribution of the residuals showed no underlying trend (Fig. 5B, D) and it was similar to that observed with the validation data (Fig. S7A, B).

A subset of 21 wheat landraces and 2 elite wheats at a similar phenological stage were selected for a second measurement along with sampling to determine LMA and N_{area} (LS-Mex2). The model bias was -3.3% for LMA and -5.5% for N_{area}. The relative error of prediction was 11.3% for LMA and 18.2% for N_{area}, compared to 7% and 7.6%, respectively, observed for the validation data (Table 3). The residuals showed no underlying trend (Fig. 6B, D), but the ranges in the LS residuals were wider than the ranges in residuals observed for the original validation data (Fig. S7C, D).

**Prediction models using a narrower waveband**

As not all spectrometers are able to measure both the visible and SWIR wavebands, we assessed the power of PLSR to predict parameters using only 400-900nm reflectance values. Their performance was generally lower with the exception of SPAD (cf. Table 2). The $R^2$ for validation data were: N_{area} 0.83, LMA 0.79, SPAD 0.8, $V_{cmax}$ 0.57, $J$ 0.56, $V_{cmax25}$ 0.48, $V_{cmax25}$/N_{area} 0.33. This indicates that significant information would be lost for the photosynthetic traits by omitting the SWIR1 and 2 bands, which would reduce the predictive power of the PLSR models.

**DISCUSSION**

The main objective of this experiment was to test if hyperspectral reflectance could be used to predict leaf nitrogen, LMA, and photosynthetic attributes in wheat. As
hyperspectral reflectance can be measured relatively quickly, could this technique be used to screen for multiple traits and enable selection of wheat genotypes for photosynthetic traits? We based this work on a previous study conducted on aspen leaves (Serbin et al., 2012). While the models developed for aspen to predict photosynthetic attributes were unsuccessful in wheat, we were able to develop new models for a variety of leaf traits. \( N_{\text{area}} \), LMA and SPAD were the traits with the highest coefficient of determination in the predictions. To assess their robustness, models were tested with previously unseen wheat genotypes. We also discuss the possibility of using calibration from other species to predict these traits.

**Predicting \( V_{\text{cmax}} \) and \( J \)**

\( V_{\text{cmax}} \) and \( J \) are underlying biochemical traits that can be derived from CO\(_2\) response curves measured using gas exchange instruments. The two traits are usually estimated from the analysis of multiple measurements taken at different CO\(_2\) concentrations. The appeal of estimating \( V_{\text{cmax}} \) and \( J \) is that they are independent of stomatal conductance and represent the amount of Rubisco and components of the thylakoid electron transport chain, respectively (von Caemmerer, 2000). Measuring \( A:C_i \) curves to estimate \( V_{\text{cmax}} \) and \( J \) is slow. Each day the gas exchange system needs to be calibrated. Each leaf needs some time under the conditions imposed in the chamber of the gas exchange system before measurements begin, to allow stomata to open and metabolism to stabilise. Each \( A:C_i \) curve takes from 15 to 40 min, depending on the number of CO\(_2\) concentrations measured. Although faster approaches have been proposed, such as a rapid \( A:C_i \) curve (Stinziano et al., 2017) or calculations using just one CO\(_2\) concentration (De Kauwe et al., 2016), these methods have not been proven in high throughput screening of genetic material under field conditions.

By comparison to gas exchange measurements, hyperspectral reflectance using the ASD Field Spec is quick to calibrate before starting and took from 15 to 50 seconds to measure a wheat leaf, depending on the settings. We found that a white reference calibration was not required before every measurement. From our experience in the field, a hyperspectral reflectance measurement was quicker to make than gas exchange measurements at a single CO\(_2\) concentration. Importantly, hyperspectral reflectance has the potential to predict as many parameters as there are calibrated
models and can be used to measure hundreds of genotypes a day, as has been shown for maize (Yendrek et al., 2017).

\( V_{\text{cmax}} \) for a leaf varies with temperature. To enable comparison between studies and because we were unable to maintain a constant leaf temperature over a day due to the natural fluctuations in ambient temperature (Fig. S1), we normalised \( V_{\text{cmax}} \) to 25 °C using revised Rubisco kinetics for wheat \( (V_{\text{cmax25}} \) Silva-Pérez et al., 2017). A similar approach was used by Ainsworth et al. (2014) who measured leaf temperature immediately before reflectance measurements. When comparing observed parameter values derived from gas exchange measurements against those predicted from leaf reflectance, \( V_{\text{cmax}} \) and \( J \) both had a higher coefficients of determination than \( V_{\text{cmax25}} \) (\( R^2 = 0.74 \) and 0.71 respectively vs 0.62) (Fig. 3). This probably reflects the fact that the range in \( V_{\text{cmax}} \) (25 to 400 \( \mu \text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \)) was greater than for \( V_{\text{cmax25}} \) (23 to 280 \( \mu \text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \)). While the \( R^2 \) value was lower for \( V_{\text{cmax25}} \) compared to \( V_{\text{cmax}} \), the relative error of prediction was also smaller (Table 2), suggesting that using \( V_{\text{cmax25}} \) is more accurate. The reflectance spectrum should be representative of the leaf composition, hence the “capacity” of the leaf, rather than the rate of the reaction per se. Another factor that could contribute to the disparity between \( V_{\text{cmax}} \) and \( V_{\text{cmax25}} \) models is if the temperature of the leaf during reflectance measurements affects the spectra. The leaf clip assembled as the factory default warms up due to the high photon flux from the internal lamp and this in turn warms the leaf during measurement. We did not observe a drift in spectral properties with sequential groups of scans which would have been associated with warming of the leaf. In most of our experiments, we used a mask with a gasket and measured the spectra within 9 s to reduce the impact of the high photon flux on leaf temperature. However, additional experiments to specifically look at the influence of leaf temperature on reflectance spectra are needed to assess this.

When the residuals from the PLS analysis of \( V_{\text{cmax}} \) and \( J \) were plotted against predicted values, no trends were apparent. However, when the residuals were plotted against observed values, positive trends were evident (Fig. S4) which indicates that factors not accounted for in the models are driving variation in the traits (Fox and Weisberg, 2011). A similar trend was evident in the prediction of \( V_{\text{cmax}} \) in maize (Yendrek et al., 2017). Despite this limitation, the results show that leaf
reflectance could be used to rank genotypes and select tails for $V_{cmax25}$ from large populations. It would then be feasible to measure the smaller numbers of genotypes in the tails using gas exchange or other more laborious approaches for confirmation. As reflectance measurements are non-destructive, this facilitates making more measurements during the plant life cycle and on more leaves within plants, which could reduce error associated with variation in plant phenology and environmental effects when assessing genotypic variation of $V_{cmax25}$ and $J$. In addition, reflectance using imaging spectroscopy has also shown promise for predicting $V_{cmax}$ at the canopy level (Serbin et al., 2015), which would provide an opportunity for canopy level high throughput estimation of photosynthetic parameters.

As with $V_{cmax}$, $J$ varies with temperature (June et al., 2004; Silva-Pérez et al., 2017). However, because the temperature response is known to vary as plants acclimate to their growth temperature (Bernacchi et al., 2003), we chose not to assume a single temperature function across experiments to derive values for $J$ at a common temperature. Caution is needed if using the current model for $J$ when phenotyping. An improved model could be created if one had access to more calibration data collected at a single temperature.

$V_{cmax25}/N_{area}$ was calculated from the data obtained here as a possible estimate of photosynthetic efficiency (ie photosynthetic capacity per unit N invested at a leaf level). Interestingly, $V_{cmax25}/N_{area}$ when treated as a trait was predicted with a higher coefficient of determination directly than by predicting each component trait separately and then calculating the ratio. It may be that the $N_{area}$ and $V_{cmax25}$ had an additive effect in training the model more accurately. While the coefficient of determination was at the lower end of the traits examined, $V_{cmax25}$ was normalized for temperature and then for leaf nitrogen, so that an $R^2$ of 0.49 (Fig. 4), would still present an opportunity to explore genetic variation in this parameter. It presumably reflects variation in Rubisco kinetic properties and activation state (assumed to be constant), mesophyll conductance (as we assumed a common function for all genotypes) and N allocation at the leaf level.

**Predicting $A$ and $g_s$**

$A$ and $g_s$ have been positively co-related with wheat yield (Fischer et al., 1998) and
are traits that need to be considered in selection of high yielding wheat genotypes. However, spot measurements of these parameters are sensitive to environmental effects.

Although light-saturated photosynthesis at ambient CO₂ has previously been predicted in trees using leaf reflectance and transmittance ($R^2=0.74$) (Doughty et al., 2011), this is surprising since $g_s$ can vary dynamically and its impact on the reflectance spectrum is unknown. When we examined our data, models predicting $A$ and particularly $g_s$ were weak ($A$, $R^2=0.49$, $g_s$, $R^2=0.34$), with $g_s$ having the greatest relative error of prediction (REP, Table 2). Both traits can change quickly in response to clouds, fluctuating temperatures or in windy conditions, but the extent that this alters reflectance spectra has not yet been determined in wheat.

Other methods, such as infrared thermography, offer a better alternative to assess stomatal conductance in the canopy, as shown under water stress and salinity tolerance (Jones, 2007; Jones et al., 2009; Sirault et al., 2009; Munns et al., 2010). Hand-held IR thermometry predicted $g_s$ under irrigated field conditions (Amani et al., 1996) and IR imaging increased accuracy and throughput (Tattaris et al., 2016). The advantage of thermography is that many plots can be compared simultaneously when imaged from above. However, variation in canopy height can confound the interpretation (Rebetzke et al., 2013). At the leaf level, the hand-held air-flow porometer (Fischer et al., 1998; Rebetzke et al., 2001) has been demonstrated to be a rapid and effective instrument to estimate $g_s$.

**Predicting $N_{area}$, LMA and SPAD**

Higher coefficients of determination and lower relative error of predictions were observed in the validation data for $N_{area}$ and LMA compared to photosynthetic traits (Table 2). This agrees with measurements collected from multiple environments, nitrogen levels and different wheat species by Ecarnot et al., (2013). The results from the current study are important since the plants evaluated were high yielding wheat and triticale, many of which are currently used by farmers around the world.

SPAD was used in this study as a “trait” because it is quick and easy to deploy in the field and could be compared with predictions derived from hyperspectral reflectance. During the data validation (Fig. 2E, $R^2=0.82$) and in experiments with different wheat
populations (Fig. 5), strong positive correlations were observed between measured and predicted SPAD values, in agreement with biochemical extraction (Doughty et al., 2011) or from Chlorophyll Normalized Difference Index (Chl NDI) (Dillen et al., 2012).

Predicting traits from reflectance measured in diverse sets of wheat genotypes

Models derived from aspen and cotton leaves were able to predict leaf nitrogen concentration and LMA from reflectance measurements on soybean (Ainsworth et al., 2014), suggesting that these models are robust across a range of species. However, the model predicting LMA with 22 wavelengths and an intercept for aspen trees (Serbin et al., 2012) gave variable results for wheat (Fig. S6). While most of the experiments could be predicted with the aspen LMA model, data measured in the field in Mexico could not. The possibility of developing a robust model to predict LMA across diverse species is appealing and published results show some promise (Heckmann et al., 2017). Here we tested our models with wheat genotypes that had not been used to develop the models for SPAD, LMA and Narea (Fig. 5 and 6). The relative error of prediction increased for this material, but as more calibration data becomes available, one would expect that the predictive ability for LMA would improve.

Models for leaf nitrogen concentration and \( V_{cmax} \) (Fig. S6) from aspen (Serbin et al., 2012) did not predict these traits in wheat. In this study, a mask (Fig. S2) was used in the leaf-clip of the ASD Field Spec to narrow the aperture so that all the wheat leaves filled the field of view. It is possible that this change in measurement geometry affected the comparison. Transferability of carbon:nitrogen ratio models between two Brassicaceae genera was poor and the performance of photosynthetic trait models was less accurate when applied to a species that had not been used to construct the model (Heckmann et al., 2017). Each model needs to be validated for the species of interest.

In general, the predictions obtained in this study for wheat were higher or within the range of \( R^2 \) for predictions of similar traits that have been reported for other species using hyperspectral leaf reflectance (Table 4). Validations for different species shown in Table 4 indicate which traits can be predicted well using hyperspectral leaf reflectance and whether they apply across species or not. Variation in kinetic
parameters for \( V_{\text{cmax}} \) between species may not be evident in the reflectance spectra. In contrast, LMA or leaf nitrogen might be more robust traits that can be predicted from a single reflectance model applied to different species.

**Training set size and source of variation**

Each hyperspectral reflectance generates 2000 values that are used to calculate each trait. PLSR solves the problem of dimensionality and multicollinearity and the issue of overfitting is dealt with by using the lowest PRESS or RMSE to determine the number of components to be used (Geladi and Kowalski, 1986). However, the question of how many observations are needed to train the model remains. In maize, 80% of the observations were used to train the model (Yendrek *et al.*, 2017). In Brassica a subset size of 90 observations and in maize 30 observations resulted in the lowest RMSE (Heckmann *et al.*, 2017). With wheat, we used about 55% of the observed data for training: 282 measurements were used to build the model to predict LMA, \( N_{\text{area}} \) and SPAD. Ecarnot *et al.*, (2013) used reflectance to predict LMA and \( N_{\text{area}} \), using a calibration obtained from a diverse collection of wheats measured under multiple conditions and environments (176 – 601 leaves). The calibration for aspen required 78 observations (Serbin *et al.*, 2012). In both of these studies, environmental treatment was a stronger driver of variation than genetic variation and the wide range of values improved the fit. Further analyses comparing the impact of training set size and range in the spectral data used to construct the models are required.

**Advantages of using hyperspectral reflectance**

The data presented here suggests that the models we obtained provide robust estimates for six different traits from a single hyperspectral reflectance measurement. Approximately 100 plants could be measured per hour in the field using the hyperspectral reflectance technique described in this study (two people measuring one plant per 6 m long plot in the field). Screening leaf physiological and biochemical parameters using this approach will enable larger populations to be analysed for photosynthetic characters which can be combined with molecular markers and genomic sequence to find regions in the plant genome related to variation in photosynthetic performance (QTLs, quantitative trait locus).
Concluding remarks

We have demonstrated the utility of leaf hyperspectral reflectance modelling to screen large wheat germplasm sets for $V_{\text{max}25}$, $J$, SPAD, LMA, $N_{\text{area}}$ and $V_{\text{max}25}/N_{\text{area}}$; a range of photosynthetic traits not easily derived in high throughput from other methodologies. This will enable wheat researchers and breeders to rapidly identify genetic variation in germplasm for crossing, genetic mapping and identification of material for more detailed mechanistic analysis.

Supplementary information

Table S1. Inlet CO$_2$ used in each experiment to measure CO$_2$ response curves.

Table S2. Training data and test data from experiments used in the PLSR model.

Figure S1. Meteorological conditions in Obregon, Mexico and Ginninderra, Australia.

Figure S2. Measuring reflectance with the leaf clip, showing leaf orientation and mask.

Figure S3. Regression coefficients for PLSR models.

Figure S4. Residuals from Figs. 2, 3 and 4 plotted against observed data.

Figure S5. Histogram of the days after flowering (DAF) when the landraces were surveyed for reflectance.

Figure S6. Validation of predictions using reflectance with the coefficients from Serbin et al., 2012 against observed data for wheat.

Figure S7. Density plots of residuals of the predictions.

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photosynthetic CO\textsubscript{2} assimilation in leaves of C\textsubscript{3} species. Planta \textbf{149}, 78–90.


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Remote Sensing 1, 013530.


### Table 1 Summary of experiments

<table>
<thead>
<tr>
<th>Expt. a</th>
<th>Set of genotypes</th>
<th>Genotypes (repetitions)</th>
<th>Stage b (DAE)</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aus1</td>
<td>EVA(-N), (+N)</td>
<td>16 (3)</td>
<td>A (73-83)</td>
<td>$V_{cmax25}$, $J_{SPAD}$, $N_{mass}$, $N_{area}$, LMA, $P_{mass}$, $P_{area}$</td>
</tr>
<tr>
<td>Aus2</td>
<td>BYPB (-N), (+N)</td>
<td>30 (2)</td>
<td>B (48-56)</td>
<td>$V_{cmax25}$, $J_{SPAD}$, $N_{mass}$, $N_{area}$, LMA, $P_{mass}$, $P_{area}$</td>
</tr>
<tr>
<td>Aus3</td>
<td>BYPB</td>
<td>28 (4)</td>
<td>B (46-54)</td>
<td>$V_{cmax25}$, $J_{SPAD}$, $N_{mass}$, $N_{area}$, LMA, $P_{mass}$, $P_{area}$</td>
</tr>
<tr>
<td>EVA</td>
<td></td>
<td>2 (4)</td>
<td>A (62-67)</td>
<td>$V_{cmax25}$, $J_{SPAD}$, $N_{mass}$, $N_{area}$, LMA, $P_{mass}$, $P_{area}$</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td>21 (4)</td>
<td>A (60-67)</td>
<td>$V_{cmax25}$, $J_{SPAD}$, $N_{mass}$, $N_{area}$, LMA</td>
</tr>
<tr>
<td>Mex1</td>
<td>CB</td>
<td>30 (3)</td>
<td>B (67-82)</td>
<td>$SPAD$, $N_{mass}$</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td>30 (3)</td>
<td>A (88-103)</td>
<td>$V_{cmax25}$, $J_{SPAD}$, $N_{mass}$, $N_{area}$, LMA</td>
</tr>
<tr>
<td>Mex2</td>
<td>CC</td>
<td>223 (2)</td>
<td>A (101-103)</td>
<td>SPAD</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td>230 landraces 40 elite wheat</td>
<td>A (110-111)</td>
<td>SPAD</td>
</tr>
<tr>
<td>LS</td>
<td></td>
<td>23 landraces 2 elite wheat</td>
<td>A (117)</td>
<td>$N_{area}$, LMA</td>
</tr>
</tbody>
</table>


b) Stage A: Anthesis, B: Booting (before anthesis). DAE: Days after emergence.
Table 2 Statistical parameters of the PLS model validation data set. Tr, training set; Val, validation or test data; NC, number of components; RMSEP-CV, root mean square error of prediction - CV, cross validation (from PLSR); REP, relative error of prediction.

<table>
<thead>
<tr>
<th>Traits</th>
<th>N Tr</th>
<th>N Val</th>
<th>RMSEP-CV</th>
<th>NC</th>
<th>R² Tr</th>
<th>R² Val</th>
<th>REP-Val (%)</th>
<th>Bias-Val (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N_area</td>
<td>282</td>
<td>243</td>
<td>0.22</td>
<td>21</td>
<td>0.92</td>
<td>0.93</td>
<td>7.6</td>
<td>0.73</td>
</tr>
<tr>
<td>LMA</td>
<td>282</td>
<td>243</td>
<td>4.50</td>
<td>21</td>
<td>0.86</td>
<td>0.89</td>
<td>7.0</td>
<td>-0.23</td>
</tr>
<tr>
<td>SPAD</td>
<td>342</td>
<td>272</td>
<td>3.16</td>
<td>16</td>
<td>0.87</td>
<td>0.81</td>
<td>6.6</td>
<td>-0.34</td>
</tr>
<tr>
<td>V_{cmax}</td>
<td>262</td>
<td>226</td>
<td>31.53</td>
<td>23</td>
<td>0.79</td>
<td>0.74</td>
<td>18.7</td>
<td>0.20</td>
</tr>
<tr>
<td>J</td>
<td>262</td>
<td>226</td>
<td>25.44</td>
<td>18</td>
<td>0.82</td>
<td>0.70</td>
<td>13.0</td>
<td>-0.73</td>
</tr>
<tr>
<td>N_{mass}</td>
<td>342</td>
<td>273</td>
<td>3.30</td>
<td>24</td>
<td>0.86</td>
<td>0.70</td>
<td>10.5</td>
<td>1.3</td>
</tr>
<tr>
<td>P_{mass}</td>
<td>219</td>
<td>212</td>
<td>0.93</td>
<td>19</td>
<td>0.54</td>
<td>0.65</td>
<td>25.8</td>
<td>3.3</td>
</tr>
<tr>
<td>V_{cmax25}</td>
<td>262</td>
<td>226</td>
<td>20.68</td>
<td>18</td>
<td>0.76</td>
<td>0.62</td>
<td>15.9</td>
<td>0.17</td>
</tr>
<tr>
<td>A</td>
<td>307</td>
<td>253</td>
<td>3.93</td>
<td>15</td>
<td>0.64</td>
<td>0.49</td>
<td>17.7</td>
<td>0.49</td>
</tr>
<tr>
<td>V_{cmax25}/N_{area}</td>
<td>262</td>
<td>226</td>
<td>10.62</td>
<td>14</td>
<td>0.40</td>
<td>0.48</td>
<td>17.5</td>
<td>1.9</td>
</tr>
<tr>
<td>P_{area}</td>
<td>219</td>
<td>212</td>
<td>0.04</td>
<td>19</td>
<td>0.40</td>
<td>0.42</td>
<td>23.5</td>
<td>4.2</td>
</tr>
<tr>
<td>g_s</td>
<td>307</td>
<td>253</td>
<td>0.15</td>
<td>11</td>
<td>0.50</td>
<td>0.34</td>
<td>33.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>

The lowest RMSEP-CV was used to choose the number of components in the model.
Table 3 Statistical parameters assessing further the models obtained in Table 2, using an independent set of wheat genotypes (elite and landraces). NC, number of components; N, number of observations; REP, relative error of prediction.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Trait</th>
<th>NC</th>
<th>N</th>
<th>$R^2$</th>
<th>REP (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-Mex2</td>
<td>SPAD</td>
<td>16</td>
<td>448</td>
<td>0.34</td>
<td>7.4</td>
<td>-3.5</td>
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<tr>
<td>L-Mex2</td>
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<td>16</td>
<td>270</td>
<td>0.44</td>
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<td>-2.3</td>
</tr>
<tr>
<td>LS-Mex2</td>
<td>LMA</td>
<td>21</td>
<td>52</td>
<td>0.14</td>
<td>11.3</td>
<td>-3.3</td>
</tr>
<tr>
<td>LS-Mex2</td>
<td>$N_{\text{area}}$</td>
<td>21</td>
<td>52</td>
<td>0.05</td>
<td>18.2</td>
<td>-5.5</td>
</tr>
</tbody>
</table>
Table 4 Comparison of the coefficients of determination ($R^2$) for leaf traits taken from publications and this paper.

<table>
<thead>
<tr>
<th>Plant material and source</th>
<th>$V_{cmax}$</th>
<th>$V_{cmax25}$</th>
<th>$J$</th>
<th>LMA</th>
<th>$N_{mass}$ (%)</th>
<th>Chlorophyll</th>
<th>$A_{400}$</th>
<th>$A_{1500}$</th>
<th>$A_{2000}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>159 tropical plants (Doughty et al., 2011)</td>
<td>0.39</td>
<td>0.52</td>
<td>0.9</td>
<td>0.83</td>
<td>70.66 (Chl a)</td>
<td>0.74</td>
<td>10.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspen, Cotton (Serbin et al., 2012)</td>
<td>0.89</td>
<td>0.93</td>
<td>0.95</td>
<td>0.89</td>
<td>70.67 (Chl b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat (Ecarnot et al., 2013)</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean (Ainsworth et al., 2014)</td>
<td>0.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize (Yendrek et al., 2017)</td>
<td>0.65</td>
<td>0.68</td>
<td>0.96</td>
<td>0.85</td>
<td></td>
<td></td>
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<tr>
<td>Brassica</td>
<td>0.55</td>
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<td></td>
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<tr>
<td>Moricandia</td>
<td>0.59</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize (Heckmann et al., 2017)</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>This study Wheat/Triticale</td>
<td>0.74</td>
<td>0.71</td>
<td>0.89</td>
<td>0.7</td>
<td>0.81</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$A_{400}$, $A_{1500}$ and $A_{2000}$, Assimilation rate measured at 400, 1500 and 2000 µmol CO$_2$ mol$^{-1}$ inlet CO$_2$ respectively.
FIGURE LEGENDS

Fig. 1 A) Reflectance from Aus1, Aus2, Aus3 and Mex1 experiments (n=565) from 400 to 2400 nm. The solid bold line is the mean and the range is given by the upper and lower solid lines. B) Loadings and C) regression coefficients of the model for $V_{cmax25}$ with 18 components.

Fig. 2 Validation of predictions (A, C, E) and residuals (B, D, F) for $N_{area}$ (21 components), LMA (21 components) and SPAD (16 components). Symbols show only the validation data, i.e. those which were not used to construct the models. See Table 2 for details.

Fig. 3 Validation of predictions (A, C, E) and residuals (B, D, F) for $V_{cmax}$ (23 components), $V_{cmax25}$ (18 components) and $J$ (18 components). Symbols show only the validation data, i.e. those which were not used to construct the models. See Table 2 for details.

Fig. 4 A) Validation of predictions and B) residuals for $V_{cmax25}/N_{area}$ (13 components). Symbols show only the validation data, i.e. those which were not used to construct the models. See Table 2 for details.

Fig. 5 Comparison of SPAD predicted from reflectance using the model developed in this study (Fig. S3) and actual SPAD measurements for elite wheat (CC-Mex2, open diamonds, a, B) or the wheat landraces set (L-Mex2, open squares, C, D) and with their respective residuals (B, D). The dashed line represents the 1:1. CC, n=448, L, n=270 and Val data, n=272. Closed circles are the validation data from Fig. 2E.

Fig. 6 Comparison of predictions using the reflectance models for LMA (A) and $N_{area}$ (C) against observed data for wheat landraces (LS-Mex2, open squares). The respective residuals are shown in (B) and (D). LS, n=52 and Val data, n=243. Closed circles are the validation data from Fig. 2A for $N_{area}$ and Fig. 2B for LMA.
FIGURES

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