Title

Image-based leaf SPAD value and chlorophyll measurement using a mobile phone: enabling accessible and sustainable crop management.

Authors

John Anderson [1]; Kulaporn Boonyaves [4]; Haruko Okamoto [1]; Netiya Karaket [5]; Wannisa Chuekong [3]; Miguel Garvie [1]; Napason Chabang [3]; Daniel Osorio [1*]; Kanyaratt Supaibulwatana [2, 3]

Address

1. School of Life Sciences, University of Sussex, Falmer Campus, Brighton, BN1 9QG, United Kingdom
2. Department of Biotechnology, Faculty of Science at Mahidol University, Bangkok 10400, Kingdom of Thailand
3. School of Bioinnovation and Biobased Product Intelligence, Faculty of Science at Mahidol University, Bangkok 10400, Kingdom of Thailand
4. Department of Biology, Faculty of Science at Mahidol University, Bangkok 10400, Kingdom of Thailand
5. Division of Agricultural Science, School of Interdisciplinary Studies, Mahidol University Kanchanaburi Campus, Saiyok, Kanchanaburi, 71150, Kingdom of Thailand

Corresponding Author

Daniel Osorio: d.osorio@sussex.ac.uk

Competing Interests

Daniel Osorio and John Anderson are co-inventors on an international patent PCT/GB2006/000639, which covers the mobile phone imaging technology used in this study. Daniel Osorio, John Anderson, and Miguel Garvie are co-owners of the PhotoFolia mobile application, which was employed in the research. The other authors declare no competing interests.

ABSTRACT

This study evaluates a practical, low-cost solution for image-based leaf SPAD (Soil and Plant Analysis Development) value and chlorophyll content monitoring using a mobile phone. We compare laboratory assay and SPAD-502+ measurements with image-based estimates from a mobile phone app (PhotoFolia). Performance is tested for four commercial rice varieties grown in Thailand. Results show that the image-based method can predict SPAD values within ± 1.2 units Mean Absolute Error (MAE) and chlorophyll concentrations within 7.2% Mean Absolute Percentage Error (MAPE) of laboratory results. Achieving a SPAD value error close to the industry standard of ±1 unit and a relative error of less than 10% in chlorophyll concentration estimation (compared to a laboratory method) demonstrates that an image-based approach using standard mobile phones can serve as an accessible, low-cost tool for on-farm chlorophyll monitoring, without the need for specialised equipment.

INTRODUCTION

Leaf chlorophyll concentration is a critical indicator of plant health. The strong correlation with soil nitrogen levels (Evans 1989) means that it provides valuable insight into nutrient status, stress, and overall productivity of plants. Monitoring chlorophyll levels enables farmers to optimize fertilizer use, improve crop yields, and detect early signs of environmental stress or nutrient deficiency (Peng 1996). Effective chlorophyll monitoring in agricultural settings requires tools that offer ease of use, speed, accuracy and affordability.

Several methods are available for measuring chlorophyll concentration, ranging from colour-matching cards (IRRI 2005) to laboratory-based assays (Table 1). Each technique offers distinct advantages and limitations, making it suited to specific applications. While laboratory-based assays provide high precision, they are time-consuming, expensive, and require specialized expertise. Portable devices like the Minolta SPAD-502+ meter offer rapid field measurements from intact leaves but are prohibitively costly for many farmers. Furthermore, interpreting SPAD readings often requires calibration tailored to specific crops or conditions, adding complexity to their use (Markwell et al. 1995). While colour cards are low-cost their application benefits from experience, and results can suffer from human bias. Variations in illumination are a problem for image-based approaches, and mobile applications often integrate additional hardware to control illumination (Agarwal 2021), which adds to their cost. Lastly, purely software-based mobile applications for chlorophyll assay offer the potential for widespread adoption due to their accessibility and ease of use, but they are regarded as the least precise (Kulig 2024)

Strategy	**Speed**	**Ease of Use**	**Cost**	**Field Usability**	**Precision**
Colour Cards[1]	Moderate	Very Easy	Very Low	Excellent	Low to Moderate
Laboratory Assay[2]	Slow	Challenging	High	Poor	Very High
Chlorophyll Meter[3]	Fast	Easy	Moderate	Excellent	High
Phone Apps, add hardware[4]	Fast	Easy	Low	Excellent	Moderate
Phone Apps, software only[5]	Fast	Extremely Easy	Very Low	Excellent	Moderate

Table 1 Comparison of chlorophyll measurement techniques

Most chlorophyll measurement techniques exploit the fact that chlorophyll absorbs light at specific wavelengths. Laboratory assays extract chlorophyll using solvents and infer chlorophyll concentration from measured absorption spectra. Portable meters like the SPAD-502+ estimate chlorophyll content non-destructively by comparing light transmitted through a leaf at a wavelength absorbed by chlorophyll relative to one that is not absorbed. Chlorophyll measurement can also take advantage of the light reflected by leaves. This is because reflected and transmitted light have similar spectral characteristics due to scattering by air-tissue interfaces in the mesophyll (Xu and Ye 2023). Remote sensing may employ hyperspectral cameras to record the spectral signatures of plants across large areas, enabling the mapping of chlorophyll and other pigments (Tucker 1979; Zimmermann 2007). The rich structure of reflectance data has enabled the development of models of leaves that predict leaf properties from spectral images (Shi 2024). The ability of algorithmic approaches to enhance the value of raw measurement data has led to software becoming increasingly important in chlorophyll monitoring. In addition, software-driven solutions, particularly those deployed via cloud services, are

[1] http://www.knowledgebank.irri.org/step-by-step-production/growth/soil-fertility/leaf-color-chart
[2] Arnon DI (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol 24, 1-15.
[3] https://sensing.konicaminolta.eu/mi-en/products/colour-measurement/chlorophyll-meter/spad-502plus
[4] Agarwal, A., Dongre, P.K. & Dutta Gupta, S. Smartphone-assisted real-time estimation of chlorophyll and carotenoid concentrations and ratio using the inverse of red and green digital color features. *Theor. Exp. Plant Physiol.* **33**, 293–302 (2021).
[5] https://www.petiolepro.com/

inherently scalable at low-cost compared to hardware systems, which depend on specialized supply chains and distribution networks. This creates an incentive to develop monitoring strategies that do not rely on specialised hardware. Notably, ordinary cameras that produce RGB signals separate reflected light into red, green and blue components which the human eye interprets as colour. From these wavelength dependent responses, advances in image-processing and machine learning have made it feasible to treat chlorophyll measurement as an inference problem.

The challenge has been to develop effective ways of mapping the camera signal onto chlorophyll concentration. Several studies have demonstrated consistent relationships between camera responses to leaves and chlorophyll concentration (Ali 2012). However, many applications require careful control of the way that the leaf is illuminated, either using a known light source, such as the camera flash, in conjunction with a specialised hardware enclosure (Agarwal 2021) or relying on contact images (Ibrahim 2021). These also require a controlled light source shining through a leaf placed over the camera aperture to ensure a consistent camera response. Despite demonstrating strong correlations between the camera response and chlorophyll concentration, the need for additional hardware limits the reach of these applications.

Purely software-based approaches do exist that attempt to quantify the greenness of leaves and relate this to chlorophyll concentration, but they do not explicitly compensate for lighting conditions or camera hardware and often rely on the use of controlled lighting conditions or a reference card to white balance the image and normalise the brightness. The Dark Green Colour Index (DGCI) first converts the camera RGB value into the HSV colorimetric space. Pixels that fall within a particular hue (H) angular range are associated with green leaf colours. These pixels are then classified based on their saturation (S) and brightness (V) to give a measure of greenness and an overall measure is derived for a given leaf area. The Green Leaf Index (GLI) is a similar measure, which involves calculating a ratio from the RGB signal that supresses red and blue signals while emphasising green. Healthy leaves containing more chlorophyll yield higher GLI and DGCI values. However, these measures are primarily used for relative comparison rather than for recovering absolute chlorophyll levels.

Despite the growing availability of such indices and related mobile applications, there remains a lack of robust validation for image-based algorithmic methods when benchmarked against conventional specialist hardware solutions such as SPAD meters or spectrophotometers. This may reflect the relatively low barrier to entry for app development, where the low cost of deployment often leads to lower user expectations for accuracy or consistency. Consequently, developers may have little incentive to conduct rigorous performance testing or calibration across different lighting conditions or device types.

There is also justification for scepticism about whether subtle variations in leaf colour can be objectively measured and reliably linked to chlorophyll concentration using images captured by standard consumer-grade cameras. The RGB signal in most digital cameras compresses the visible spectrum into three broad spectral bands (red, green, and blue) which inherently limits spectral resolution. Moreover, this signal is influenced by external factors such as ambient illumination, camera sensor characteristics, and image processing algorithms, making consistent and objective quantification of biophysical properties like chlorophyll content challenging. As a result, while DGCI and GLI offer accessible and non-destructive proxies for assessing plant health, their reliability and reproducibility remain constrained by the limitations of the imaging systems on which they rely.

To address this knowledge gap, we compare three types of chlorophyll measurement: a standard laboratory-based assay (spectrophotometric measurement), a SPAD-502+ meter, and estimates derived from mobile phone images of leaves obtained under ambient conditions (PhotoFolia app).

MATERIALS & METHODS

Rice growth protocol

Seeds of four Thai rice, *Oryza sativa* ssp., Indica cultivars, Khao Dawk Mali 105 (KDML), RD6, RD47, and Pathum Thani 1 (PPT1) were obtained from Pathum Thani Rice Research Centre, Thailand (Jiamtaea 2017). Seeds were surface sterilised and were imbibed and kept at 30°C for 5 days to induce germination. The vigorous germinated seeds were transferred to a hydroponic-based microgreen system (AgrowLab® Co.Ltd., Thailand) supplemented with Yoshida solution (Yoshida 1976) and incubated under a 16 h d^{-1} photoperiod at 80 mmol photons m^{-2} s^{-1} of LED lighting (445, 554 nm) for three weeks. Seedlings of each of the four cultivars with uniform growth were then individually transferred to vermiculite-based substrate culture supplemented with nitrogen levels of 0.26 mM NH_4NO_3 or 2.056 mM NH_4NO_3 of modified Yoshida formulation, and placed in a Mahidol University greenhouse (Phayathai campus) with average temperature at 33.8±6.4 °C and 17,409±1,881 lux illumination (recorded by the Data logger model UA-002-64; HOBO Pendant® Temperature/Light 64K Data Logger). The experiments were set up in the greenhouse on August 14, 2024, and all parameters recorded after 21 days of cultivation on September 4, 2024.

Leaf measurement protocols

The third leaf from the shoot apical meristem of seven-week-old rice plants was harvested and approximately the same mid region of the leaf was analysed using four techniques.

Leaf images were first collected using the PhotoFolia app and colour standard. The PhotoFolia app is available for both Android and iOS operating systems (https://www.colourworker.com/photofolia-user-guide/). Three models of iPhone were used in this study (14 Pro, 14 Pro Max & 15 Pro Max). The PhotoFolia app was downloaded from the Apple (iOS) store using the QR code printed on the colour standard. After registering (Google/Apple/Facebook) the class of reference reflectance spectra (rice variety/condition) was selected before taking a photograph of the colour standard together with a rice leaf (Fig. 1a).

The colour standard is mounted on a pair of bamboo forceps enabling a leaf section to be gripped, pressed flush and aligned with a 10mm diameter circular aperture in the centre of the standard (Fig. 1a). Under uniform illumination ensures that both the leaf and the standard receive the same light.

The acquired image is uploaded to a cloud-server (AWS) running the Matlab Production Server (Mathworks) where image-processing takes place before results are returned to the mobile phone client for display. An example of the PhotoFolia app results screen (Fig. 1b) displays the sample region together with estimates of SPAD value, chlorophyll concentration and (HSV) colorimetric coordinates. Historical image data and estimates can also be browsed and downloaded from a web application that is accessible via the account used when registering the PhotoFolia app (https://colourworker.com/apps/photofolia/showmeasurements/).

Actual SPAD measurements were then obtained from approximately the same leaf region using a Minolta 502+ SPAD meter. A single measurement was recorded for each leaf sample.

To measure leaf reflectance spectra, the leaf section was mounted in a custom-built aluminium light tight enclosure (85 x 60 x 60mm) allowing the leaf surface to be diffusely lit by a Halogen fibre-optic illuminator oriented at 45° to the surface normal (Fig. 1c). The spectral distribution of reflected light was measured using an Ocean Optics Flame S-XR1 Spectroradiometer & PTFE white standard. A 200µm UV-VIS fibre optic was oriented at 22.5° to the leaf surface normal to minimise specular

reflection. The tip of the fibre optic was focused via a collimating lens to sample from an elliptical region (~5mm minor axis) on the leaf surface.

Finally, the same segment of leaf was weighed and snap frozen in liquid nitrogen for chlorophyll extraction. Chlorophyll was extracted by grinding the tissues in 80% acetone and the absorption maxima of chlorophyll a (662 nm) and b (644 nm) were measured by a spectrophotometer (Thermo-scientific Genesys 10s uv-vis spectrophotometer, METTLER TOLEDO©, USA). Total chlorophyll per gram fresh weight of each tissue was calculated by the formula published by Lichtenthaler (Lichtenthaler 1987).

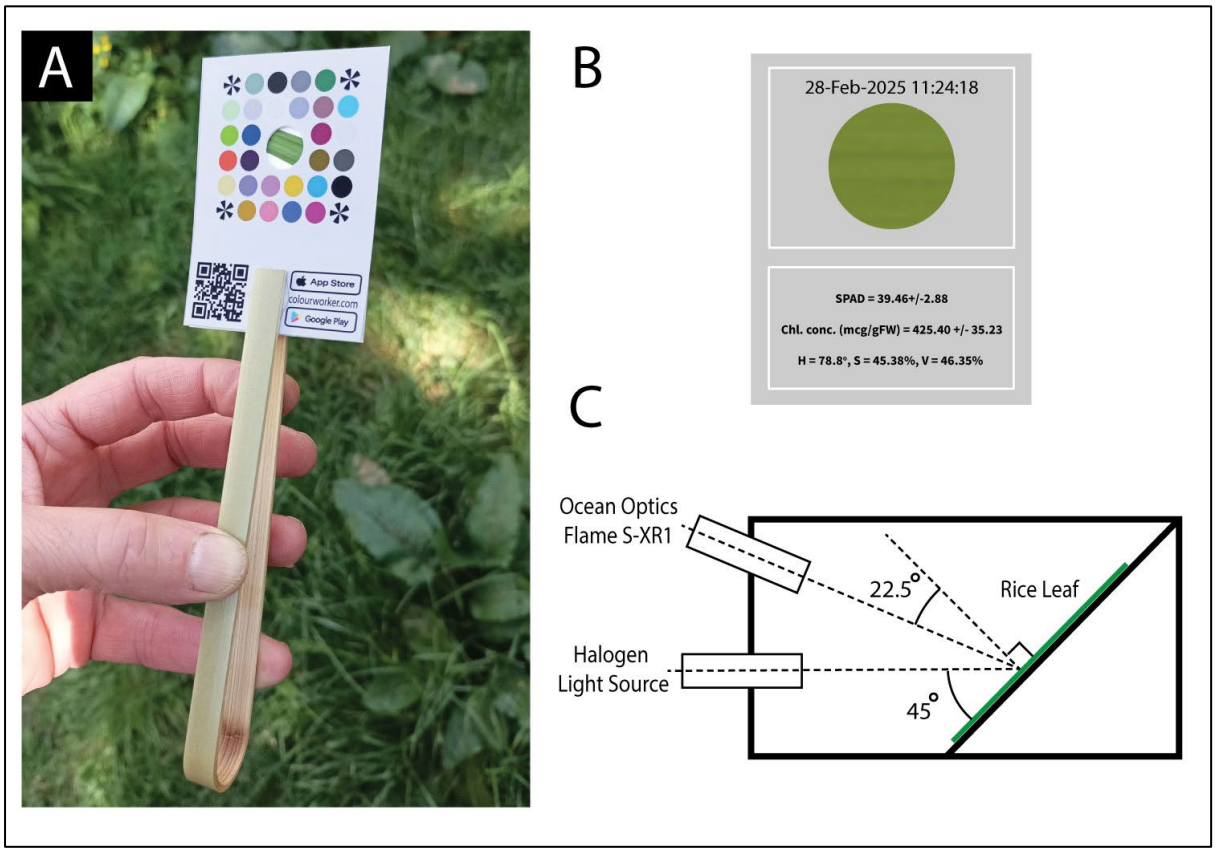

Fig. 1. a) Colour standard tweezers enable a leaf segment to be gripped flush with the surface of the standard and aligned with the sample aperture. The user takes a photograph of the colour standard from within the PhotoFolia app taking care to include the radial corner location motifs. b) PhotoFolia results screen displays the leaf sample region together with SPAD value, chlorophyll concentration and HSV colorimetric coordinates. c) Reflectance Spectrum measurement schematic. The leaf is mounted at 45° inside a light tight matt black painted aluminium housing. Illumination is via a microscope fibre optic halogen light source at 22.5° to leaf normal. Spectral reflectance measured at 45° to leaf normal via fibre optic focused with collimating lens and Ocean Optics Flame S-XR1 spectroradiometer using OceanView software.

Image-based estimates of chlorophyll concentration and SPAD value using PhotoFolia

Recovery of objective information about leaves from ordinary camera images presents two challenges. First, the camera RGB signal (C) depends on the spectral composition of the illuminant (I), the spectral reflectance of the leaf (R) and the spectral sensitivity of the camera sensor (S).

$$C_i = \int_{400nm}^{700nm} I(\lambda)R(\lambda)S_i(\lambda)d\lambda \qquad i = [R, G, B] \qquad \text{eqn. 1}$$

This relationship means a leaf photographed with a different make/model of camera or under different lighting conditions will produce a different camera signal. It is therefore necessary to compensate the camera response to make it invariant to the illuminant and to camera spectral sensitivity while preserving information about changes in leaf spectral reflectance. The second challenge is that although changes in chlorophyll concentration influence the spectral reflectance of the leaf, understanding how the camera responds to these changes is necessary to infer chlorophyll levels from the camera signal. The PhotoFolia app address both of these challenges (Fig. 2) to enable an objective measure of chlorophyll content (or SPAD value) to be recovered from leaf images.

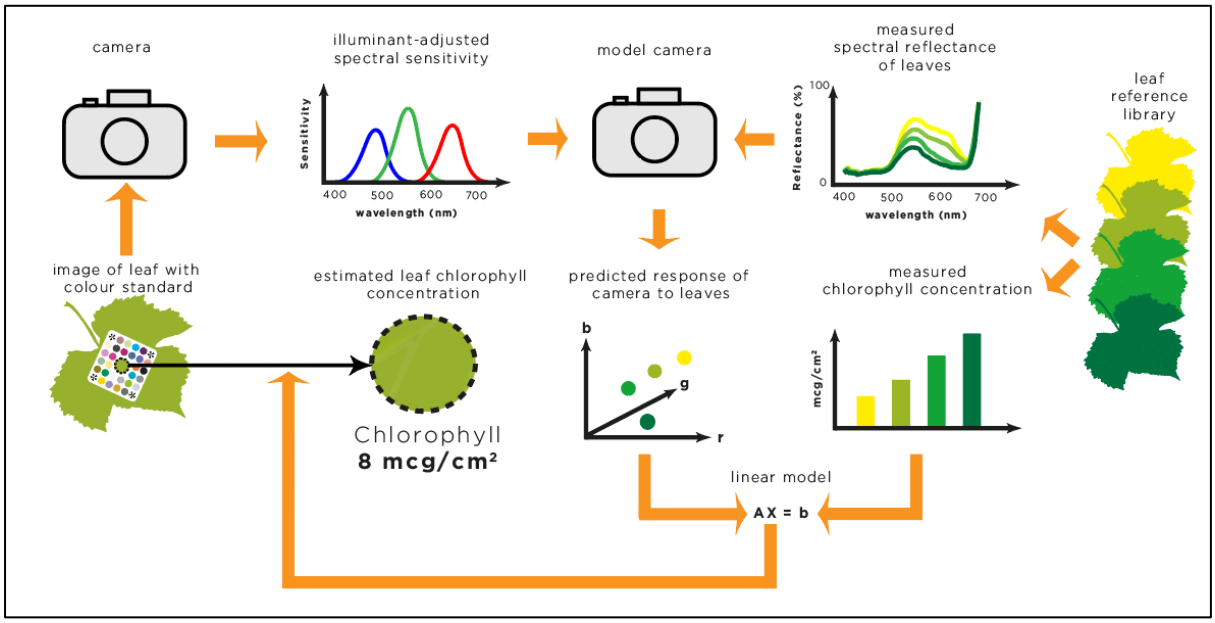

Fig. 2. Schematic of algorithm for chlorophyll measurement implemented by the PhotoFolia app. Left side: a real image of a leaf containing a colour standard with coloured patches of known spectral reflectance is used to estimate a model of the spectral sensitivities of the camera's red, green and blue sensors that also takes account of the scene illumination. Centre: the model camera is used to predict the response of the real camera to a set of reference reflectance spectra of leaves containing different levels of chlorophyll (right). Bottom-right: measured leaf chlorophyll concentrations are regressed on to the estimated model camera response to the reference reflectance spectra. This linear model is then used to predict the chlorophyll concentration from real image RGB values recovered from the leaf (bottom-left).

To compensate for variations in scene illumination and make/model of camera, PhotoFolia employs a colour standard comprised of coloured patches of known spectral reflectance. The camera RGB signals associated with the patches recovered from an image of the colour standard are used to estimate a model of the spectral encoding characteristics of each of the camera's colour channels that also takes account of the scene illumination. This is achieved by a constrained least-squares optimisation (Finlayson 1998). The resulting camera model can then predict how the real camera will respond to leaf reflectance spectra.

Given a sample of leaf reflectance spectra that are paired with SPAD or chlorophyll assay measurements it is possible to train a model to predict chlorophyll concentration or SPAD value from the estimated camera response to the leaf reflectance spectra. The resulting model can then predict SPAD value or chlorophyll levels from RGB values recovered from actual leaf images. If the variation

in the spectral reflectance of the subject material can be described using relatively few degrees of freedom it is possible to recover accurate estimates from the camera signal (Chiao et al. 2000), and makes (near) optimal use of camera RGB signals to estimate spectral reflectance - or any quantity that is correlated with spectral reflectance.

A detailed evaluation of the accuracy of camera models is beyond the scope of this study. However, the adopted quadratic programming approach (Finlayson 1998) is an effective strategy for finding robust, accurate and reproducible solutions to inherently ill-conditioned optimisation problems.

The relationship between camera response and chlorophyll concentration and SPAD value.

The relationship between chlorophyll concentration, SPAD value, and light absorbed by leaves has been extensively researched (Gausman 1985; Uddling 2007). The main concern of this study is to select an appropriate model for relating the RGB signal derived from a leaf image to SPAD value and chlorophyll concentration, which requires a brief consideration of the physical processes underlying light scattering by leaves.

The fundamental theoretical relationship between chlorophyll concentration and the absorption of light by leaves is approximated by the Beer-Lambert law, which relates light absorption to pigment density and optical path length. This means that the ratio of reflected (R) or transmitted (T) light relative to incident light (I0) that is not absorbed will be exponentially related to the thickness of the leaf (L) and the concentration of chlorophyll (C).

$$\frac{T}{I_0} \propto \frac{R}{I_0} \propto e^{-CL} \qquad \text{eqn. 2}$$

This implies that the logarithm of relative reflectance or transmittance is inversely proportional to chlorophyll concentration. The Minolta 502+ SPAD meter assumes this relationship and while the actual function employed is proprietary, containing an unknown constant (k) the published relationship (Uddling 2007) compares the amount of light transmitted at a wavelength strongly absorbed by chlorophyll (650nm) with one that is not strongly absorbed (940nm).

$$SPAD \propto \frac{T}{I_0} = k \log_{10} \frac{I_{0(650)} I_{(940)}}{I_{(650)} I_{0(940)}} \qquad \text{eqn. 3}$$

According to eqn. 2 & 3, SPAD value and chlorophyll concentration are expected to be linearly related. However, in reality the Beer-Lambert law is rarely strictly satisfied, and many empirical studies find a non-linear monotonic relationship between SPAD value and chlorophyll concentration (Markwell et al. 1995; Monje 1992).

While the assumption underlying the derivation of the SPAD-value measurement is important, we are specifically concerned with the relationship between the camera response to images of leaves, and SPAD and chlorophyll measurements. Most modern cameras employ CMOS sensors, which are linear with respect to photon arrival rates and thus light intensity. This means that the camera RGB signal (C) should be linearly related to relative changes in leaf spectral reflectance, and that the logarithm of the RGB signal should be inversely (linearly) related to SPAD value and chlorophyll concentration.

$$C \propto \frac{R}{I_0} \propto e^{-CL} \propto e^{-SPAD} \qquad \text{eqn. 4}$$

$$\log_{10} C \propto -CL \propto -SPAD \qquad \text{eqn. 5}$$

This suggests that a linear model regressing SPAD value and chlorophyll concentration onto the logarithm of the camera response is an appropriate choice. However, because the Beer-Lambert approximation may not be satisfied in practice, the relationship between predicted camera responses and SPAD values and chlorophyll concentration was briefly investigated, and linear models fitted to raw and log transformed camera data were compared.

Choice of reference reflectance spectra when making estimates using PhotoFolia.

PhotoFolia requires reference reflectance spectra to train the model for predicting SPAD values and chlorophyll concentrations from leaf images (Fig. 2). This step is critical because the model must account for the spectral characteristics of light reflected by the leaf. However, the appropriate level of specificity when selecting reference spectra requires consideration. If all leaves exhibited identical changes in spectral reflectance as a function of chlorophyll concentration, a "generic" set of reference spectra would suffice. However, leaf spectral reflectance is influenced by multiple factors beyond chlorophyll concentration, including structural features such as ridges, hairs, and surface roughness, and secondary pigments including carotenoids and anthocyanins. Furthermore, there is no universal mapping between hardware-based SPAD measurements and leaf chlorophyll content (Uddling 2007) so a single set of reference spectra may not adequately capture species-specific variations in the relationship between spectral reflectance and chlorophyll concentration.

To evaluate the choice of reference spectra on predictive error this study compared three types of reference library:

1. A generic library combining spectral reflectance measurements across all rice varieties and experimental conditions.
2. Variety-specific libraries, which included reflectance spectra from both control and treatment groups for each rice variety.
3. Variety-specific and group-specific libraries, which separated reflectance spectra by both rice variety and experimental condition.

In each case, the appropriate variety- and/or group-specific reference spectra were applied when estimating SPAD values and chlorophyll concentrations for a given rice variety and experimental condition.

PhotoFolia (training and testing)

PhotoFolia uses the reference spectra to calibrate a predictive model for estimating SPAD values and chlorophyll concentrations from image RGB values. To optimise the use of a relatively small dataset, a leave-one-out cross-validation (LOOCV) strategy was adopted for evaluating model performance. This approach maximises use of available data during training while rigorously excluding any information pertaining to the spectral reflectance, SPAD values, or chlorophyll concentrations of the test sample, thereby maintaining the integrity of the validation procedure.

The predictive model for estimating SPAD values and chlorophyll concentrations from RGB values is of the linear form b=AX, where b is an n-dimensional vector of known rice leaf SPAD values or chlorophyll concentrations, A is an n×3 matrix containing either raw RGB values or their logarithmic transformation (log(RGB)), and X represents the unknown model coefficients to be estimated. Due to

the high degree of collinearity among the responses of the camera to leaf reflectance spectra—stemming from the distributed influence of chlorophyll on spectral reflectance—a standard least-squares estimation approach may lead to unstable solutions. To address this issue, ridge regression was employed as a regularization technique to stabilise the estimation of X. The regularization parameter λ was optimized using a grid search procedure combined with 3-fold cross-validation.

Three metrics were used to evaluate the accuracy of estimated SPAD values and chlorophyll concentrations against measured values. The Mean Absolute Error (MAE) measures the average absolute difference between estimated and measured values, providing a straightforward assessment of performance that is independent of the magnitude of the values. MAE provides an intuitive sense of the typical error size in the same units as the data, making it easy to interpret. Additionally, the Mean Absolute Percentage Error (MAPE) assesses accuracy relative to the magnitude of the measurements and is a useful measure of utility, in particular identifying potential bias in estimates at low values. Finally, R-squared (R^2) values were calculated as an indication of the proportion of variance in the measured values that is explained by the estimated values. This measure is useful for assessing the overall goodness-of-fit of the model and reveals whether the estimation approach is effectively taking advantage of structure in the training data. Bland-Altman plots were also used to reveal obvious structure in the errors as a function of estimation value.

RESULTS

Camera response to leaf reflectance spectra vs measured SPAD values and chlorophyll concentration.

To comprehensively examine the relationship between the camera response, measured SPAD value and chlorophyll concentration, it is necessary to ensure controlled illumination during imaging and precise tracking of a defined spatial region of the leaf during imaging, SPAD measurement, and chlorophyll assay. A standardised protocol of this kind was not applied in this study. However, it is possible to gain insight into the relationship between the camera response and measured SPAD value and chlorophyll concentration by assuming that the camera is linear. Using eqn. 1 the response of the camera can be calculated from an estimate of the illuminant-adjusted spectral sensitivities and the measured reflectance spectra of different leaves. The calculated camera responses will approximate the real camera response to the leaves, and this can be compared to measured SPAD values and chlorophyll concentrations from the same leaves. This relationship can help to inform the most appropriate choice of model for making predictions from the real camera RGB values.

The calculated response of the green channel of a model camera to the measured leaf reflectance spectra are plotted against measured SPAD values (Fig. 3) and measured chlorophyll concentrations (Fig. 4). Because the different camera channels responses are highly correlated only the green channel is shown.

In Fig. 3 the untransformed green channel response (Fig. 3A) and the logarithm of the response (Fig. 3B) are plotted against SPAD value. The observed negative correlation between the SPAD value and the camera response is expected because the more light that is absorbed by chlorophyll in the leaf (higher SPAD value) the less light will be reflected (lower reflectance). The camera response (by definition; eqn. 1) is directly linearly proportional to spectral reflectance, which implies a logarithmic relation between SPAD value and camera response. However, there is only a weak indication of this as a slight asymptote of the camera response at high SPAD values (Fig. 3A). Logarithmically transforming the camera response does linearise this response (Fig. 3B). However, the linearity of the camera response to less green leaves (lower SPAD values) is disrupted by this transform. The benefit of logarithmically transforming the camera response was investigated by fitting linear models to

predict SPAD values given the untransformed and log-transformed camera data. Log transforming the camera response resulted in an increase in both the MAE and the MAPE from 0.78 SPAD units and 2.65% to 0.84 SPAD units & 2.80% respectively, which favours the linear model to predict SPAD value from the camera signal for real image data.

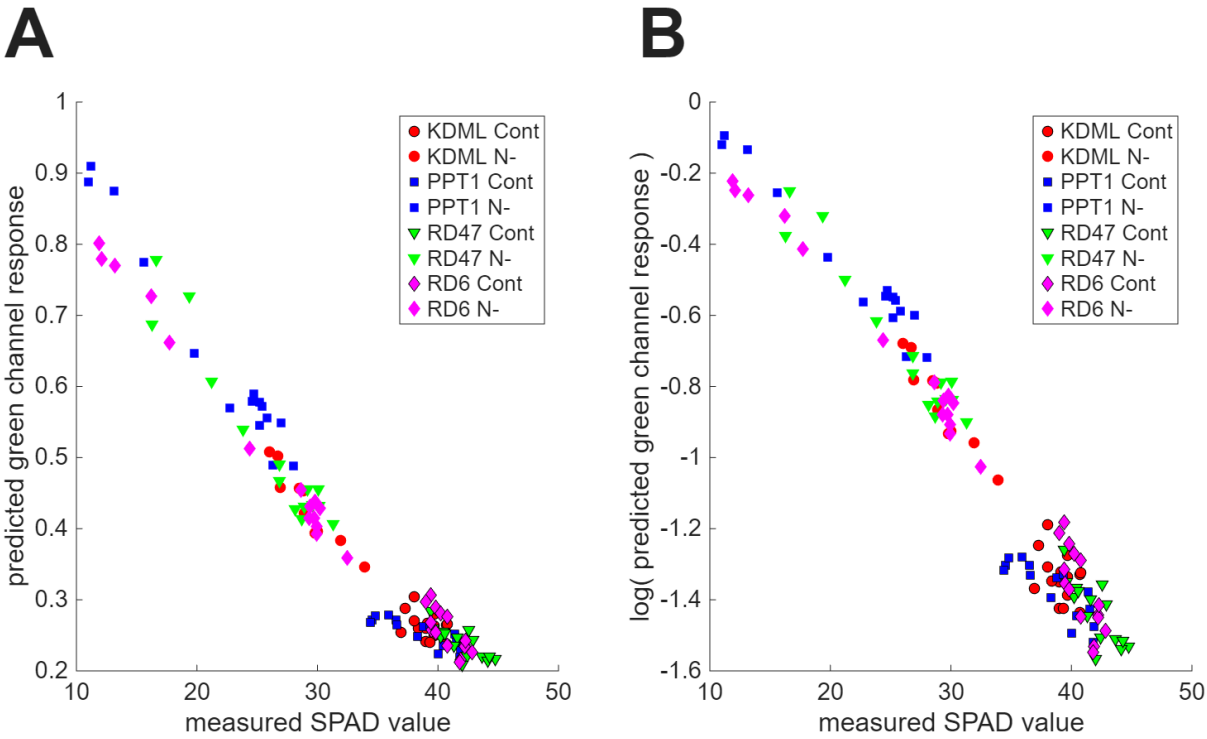

Fig. 3 Comparison of predicted green channel camera response to reflectance spectra obtained from seven-week-old leaves of KDML, PPT1, RD47, and RD6 rice varieties grown under two nitrogen concentrations Cont (2.056 mM), and N- (0.26 mM) with SPAD value measurements from same leaves. (A) camera response is inversely proportional to measured SPAD value, with an asymptote apparent at high SPAD values. (B) log transformation of the camera response linearizes the relationship at high SPAD values but results in a loss of linearity at low SPAD values.

As expected, there is a clear indication of a negative correlation between chlorophyll concentration and the predicted green channel response (Fig. 4) but the relationship between concentration and camera response depends on both rice variety and experimental treatment. Once again, there is an indication that the log transformation of the camera signal linearises the relationship at higher chlorophyll concentrations, although the control groups (Fig. 4A, black edged symbols) display a reasonable linear relation, albeit over a smaller range of green channel excitation. In addition, as for the SPAD/camera response, logarithmic transformation introduces a slight asymptote at low chlorophyll concentrations (Fig. 4B). Because of the less consistent relationship between the predicted camera response and the chlorophyll concentrations no attempt was made to fit a single linear model to this data. Instead, the mean MAE and MAPE values were calculated from models fitted to variety and condition specific data for untransformed and log transformed camera responses. As for the SPAD data, logarithmically transforming the camera response resulted in an increase in the MAE and MAPE from 26.08 ug/g FW and 4.31% to 27.03 ug/g FW & 4.37% respectively. This is a minor difference, but, as for SPAD estimates, the fact that lower MAE and MAPE were associated with using untransformed camera data favoured applying a purely linear model to predict chlorophyll concentration from the camera signal.

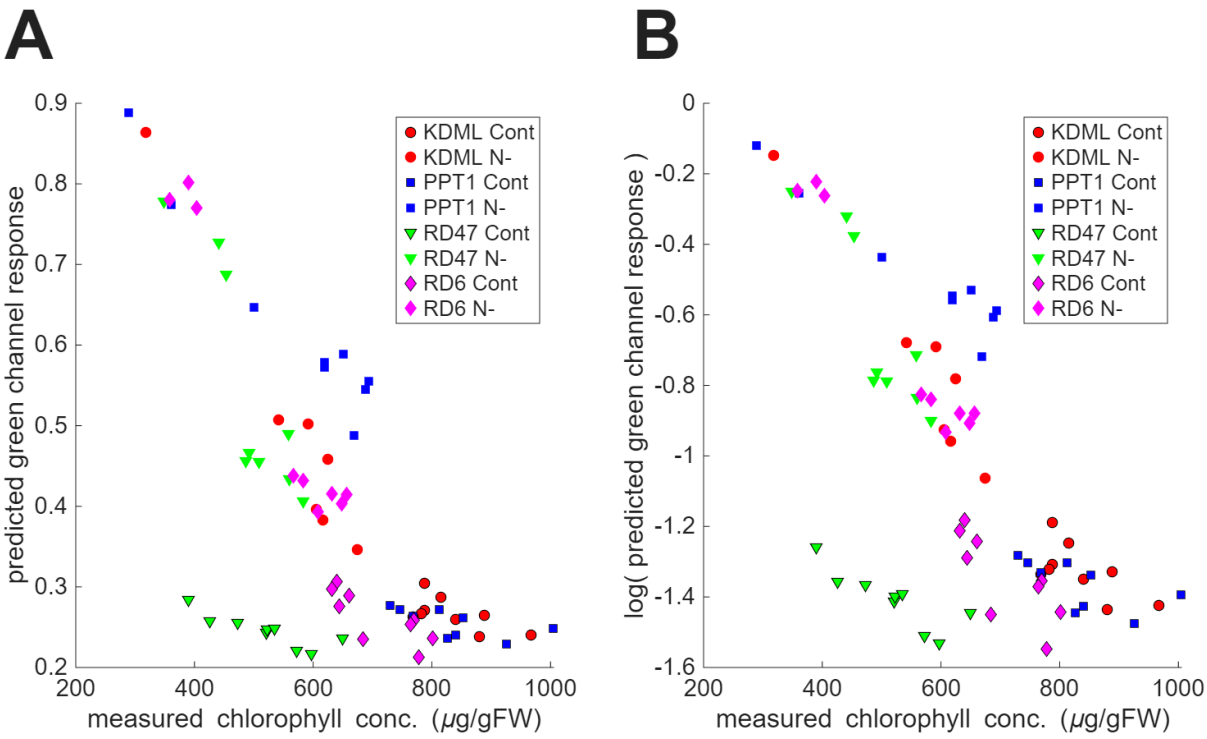

Fig. 4. Comparison of predicted green channel camera response to reflectance spectra obtained from seven-week-old leaves of KDML, PPT1, RD47, and RD6 rice varieties grown under two nitrogen concentrations Cont (2.056 mM), and N- (0.26 mM) with chlorophyll concentration measurements (μg/gFW) from the same leaves. (A) There is an inverse relationship, between the camera response and measured chlorophyll concentration, however, this is not generally consistent and depends on the rice variety and growth condition. There is some suggestion of an asymptote of the camera response in control groups at high chlorophyll concentrations although the inverse relation still holds, indicating that the camera is responding to changes in chlorophyll concentration. (B) Log transformation of the camera response largely preserves the inverse linear relationship and also slightly reduces the asymptote of the camera response at high chlorophyll concentrations observed in the control groups. However, there is some indication of the introduction of a slight asymptote at low chlorophyll concentrations.

Plotting measured SPAD value against measured chlorophyll concentration (Fig. 5) reveals the inconsistent relationship between these two quantities. While rice variety and condition separately display reasonable linear relationships indicated by linear fits and 95% confidence intervals on the slope parameter, there are noticeable variations between groups. In particular, the control and treatment groups associated with the rice variety RD47 are not continuous with one another. Similar chlorophyll concentrations in both groups are associated with quite different SPAD values. This study is not intended to provide a detailed assessment of the relationship between measured SPAD value and measured chlorophyll concentration, but these results imply that while a single generic model relating the camera response to SPAD value might be a valid choice, this is unlikely to be effective for predicting leaf chlorophyll content. This is because the variety- and condition-specific changes in chlorophyll concentration as a function of SPAD values differs substantially from the average change.

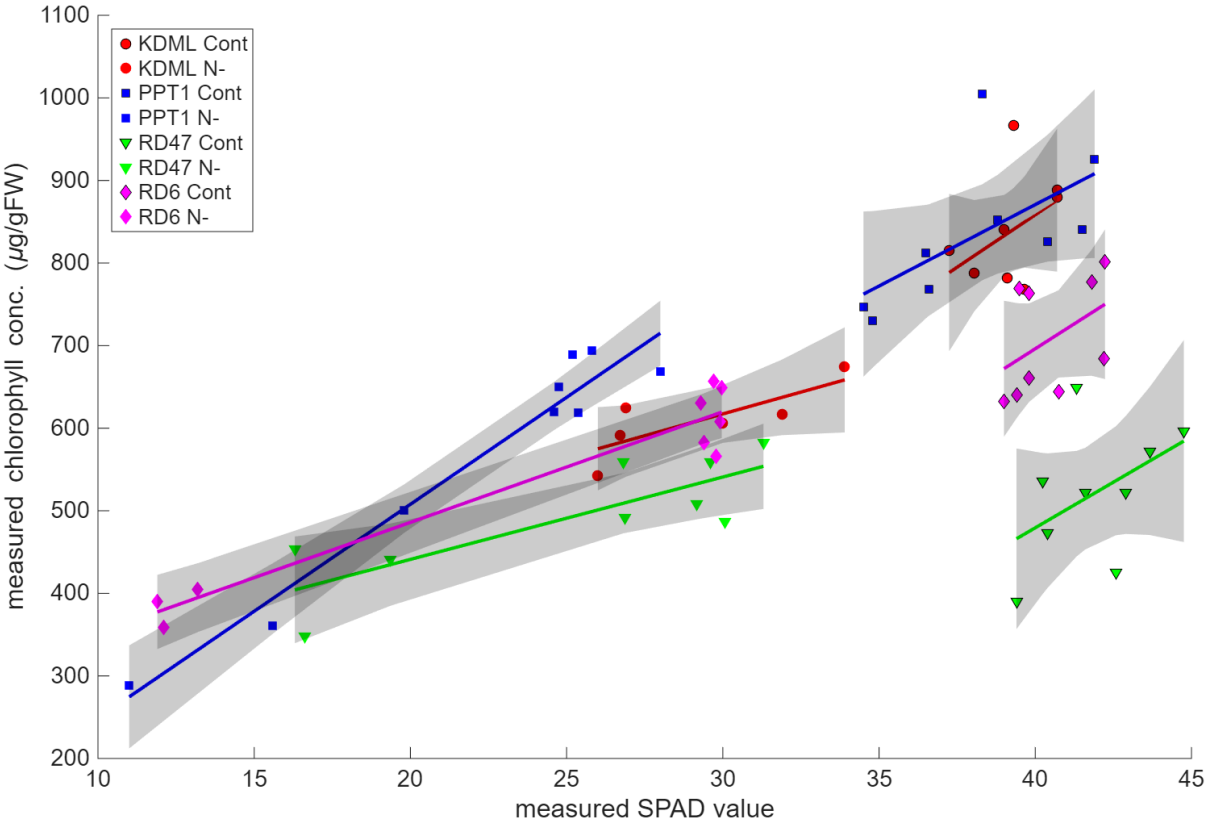

Fig. 5. Comparison of measured chlorophyll concentration (µg/gFW) and measured SPAD values from the same seven-week-old rice leaves of KDML, PPT1, RD47, and RD6 rice varieties grown under two nitrogen concentrations Cont (2.056 mM), and N- (0.26 mM). Linear fits together with 95% confidence intervals on the slope parameter are indicated in grey. All rice varieties and conditions display a positive linear correlation. The correlation is weaker in the control groups partly as a result of the reduced measurement range. There is a strong indication of variety and condition specific variations in the relationship (see text).

Choice of reference reflectance spectra when making estimates using PhotoFolia

To evaluate how the choice of reference spectra affects PhotoFolia estimates, we compared SPAD values and chlorophyll concentrations obtained from real camera images of leaves to their corresponding measured values, while varying the specificity of the reference spectra used (Fig. 6). Lines of equality are plotted in black.

The scatterplots (Fig. 6A, B, C) compare rice leaf SPAD values measured using a Minolta 502+ SPAD meter and SPAD values estimated from images of the same leaves using PhotoFolia. The scatterplot 6A results from using variety- and condition-specific reference reflectance spectra (separate treatment and controls corresponding to the most specific configuration) when making estimates. The scatterplot 6B makes use of variety-specific reference spectra (combining control and treatment spectra for each rice variety) and 6C combines all reference spectra across rice variety and condition (least specific configuration).

As reference spectra specificity decreases (left to right) MAE and MAPE and R^2 all increase but the effect is quite small. The relative error (MAPE) which is a useful summary of overall performance increases from 4.1% to 6.6% when moving from using the most specific to least specific reference spectra.

For chlorophyll estimates (Fig. 6D, E, F) the choice of reference spectra is more significant than for SPAD value. The relative error increases from 7.2% when using variety- and condition-specific

reference spectra to 22.5% when applying combined reference spectra. Using combined reference spectra also result in a negative R^2 value and there is no visual indication of a correlation between measured and estimated values.

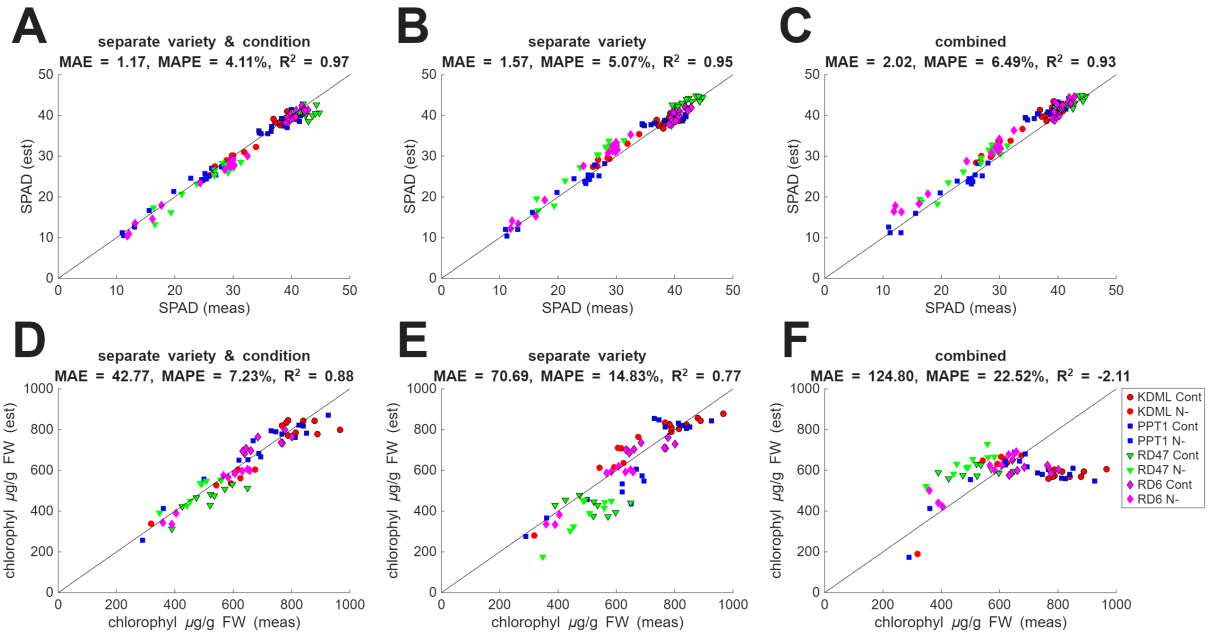

Fig. 6. Comparison of measured SPAD value (A, B, C) and chlorophyll concentration (D, E, F) with image-based estimates made using PhotoFolia app showing the impact of varying the specificity of leaf reference reflectance spectra (left to right). Seven-week-old leaves of KDML, PPT1, RD47, and RD6 rice varieties grown under two nitrogen concentrations Cont (2.056 mM), and N- (0.26 mM) were tested. (A, D) separate variety, control and treatment specific reference spectra are used. (B, E) control and treatment reflectance spectra are merged into variety-specific groups. (C, F) all reference spectra are merged into a single group. Decreasing specificity (left to right) has a small impact on SPAD value estimation accuracy. MAE increases from 1.17 to 2.02 SPAD units and estimates remain strongly correlated with measurements. In contrast, the accuracy of chlorophyll estimates decreases substantially as reference reflectance spectra specificity reduces from a MAPE of 7.23% to 22.5%. There is a complete loss of correlation between estimates and measurements when utilising the combined reference reflectance spectra.

Bland-Altman plots (Fig. 7) compare the difference between measured and estimated values as a function of average value and align to plots on Fig. 6. In the case of SPAD value there is a small but significant bias ($p<0.01$ t-test) resulting in a slightly higher estimated SPAD value compared to the measured SPAD value. This bias is lowest when applying the most specific reference spectra (0.72 SPAD units, Fig. 7A) and highest for the combined reference (1.79 SPAD units, Fig. 7C). The limits of agreement (LoA) increase as reference library specificity decreases (left to right). However, even when applying the combined reference, most estimated SPAD values lie within +/- 3 SPAD units of the measured values (Fig. 7C). There is some indication that for estimates associated with leaves belonging to the control groups, errors correlate with SPAD value, however, these errors still display relatively little bias.

For chlorophyll estimates (Fig. 7D, E, F) both bias and LoA also increase with decreasing reference reflectance spectra specificity, and the impact of reducing specificity is more pronounced than for SPAD value. When applying separate variety and condition specific reference reflectance spectra (Fig. 7E) most chlorophyll estimates fall within +/- 100 µg/g FW of known values. However, estimates using combined reference spectra (Fig. 7F) span a range of 600 µg/g FW around the measured value. In addition, the errors are highly dependent on the chlorophyll concentration.

These observations highlight the close link between leaf light transmission (SPAD) and reflection (PhotoFolia) (Xu & Ye 2023) and their less direct relationship with chlorophyll concentration.

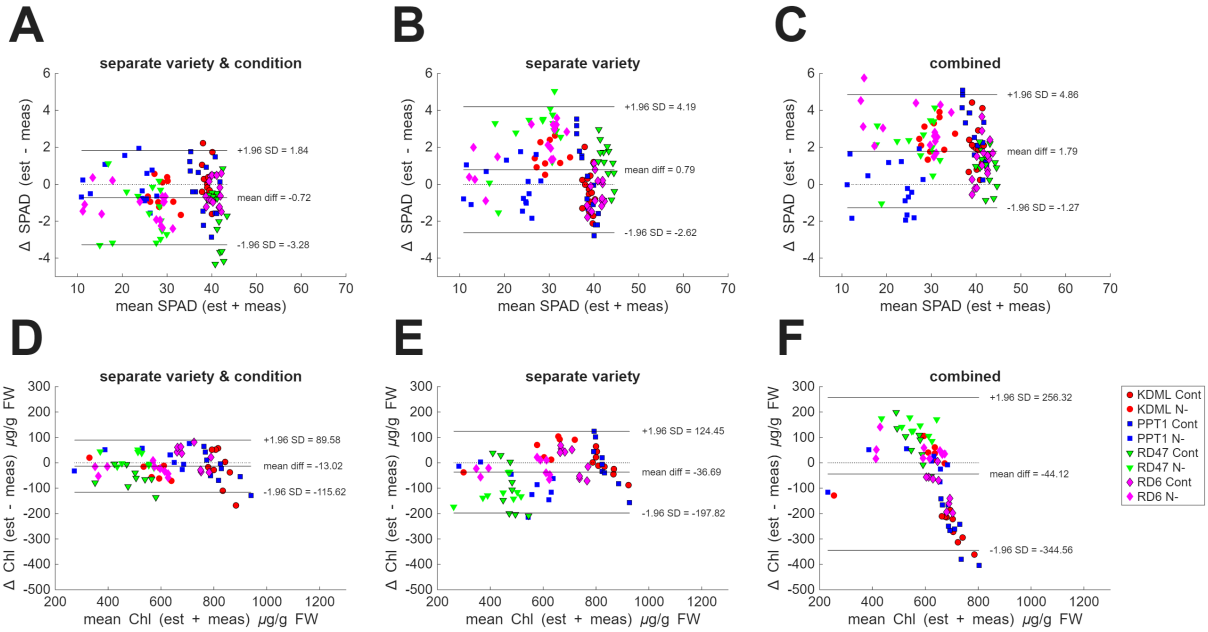

Fig. 7. Bland-Altman plots associated with PhotoFolia image-based estimates corresponding to results presented on Fig. 6 (see text for details).

Discussion

This study finds that a mobile phone-based method can predict SPAD values within +/- 1.2 SPAD units (MAE) and chlorophyll concentrations within 7.2% (MAPE) of laboratory assay results. These results suggest that a mobile phone-based solution, that employs a cardboard colour reference standard, could offer a practical and cost-effective method for informing plant management decisions in the field.

Predicting SPAD value and chlorophyll concentration from camera responses.

Whereas the Beer-Lambert law implies that the logarithm of the camera response should be linearly related to SPAD values (and chlorophyll concentrations), this model did not significantly reduce prediction errors compared to a simpler linear model. Based on predicted camera responses, a purely linear model appeared to be a reasonable and practical choice. A more complex model based on the relationship between leaf spectral reflectance, camera response, and SPAD/chlorophyll values might improve prediction accuracy, but there is no clear evidence that this relationship is especially complex, and using the simplest (linear) form is unlikely to compromise practical performance.

Accuracy of SPAD estimates

The MAE of image-based SPAD value estimates (± 1.2 units) is similar to the published accuracy of the Minolta 502+ SPAD meter (± 1.0 units). This is a reasonable expectation if the factors that are known to contribute uncertainty to image-based estimates are effectively mitigated. The variation in the relationship between measured SPAD value and PhotoFolia estimates, as represented by MAP and MAPE values (Fig. 6) might arise simply because the precise position of SPAD and PhotoFolia measurements on the leaf differed slightly. Consequently, we could not ensure that the pixel values

used for image-based estimates corresponded to the same region of the leaf covered by the SPAD meter's aperture. The measurement aperture, which is relatively small (approximately 6 mm in diameter), is ideal for measuring leaves with small areas but may, by chance, align with a leaf vein or an interveinal area, each having different light absorption properties. Although this risk was minimal for healthy rice leaves (controls), which are relatively uniform in colour, nitrogen depletion treatments caused variegation (striping) in leaf surface colour, which could easily cause the measured variations.

Accuracy of chlorophyll estimates

The situation is more complex for chlorophyll estimates. This study does not find a consistent linear relationship between SPAD value and chlorophyll concentration, based on the simplest interpretation of the Beer Lambert law. Instead, different varieties of rice leaf grown under different conditions exhibit unique (albeit linear) relationships between SPAD value and chlorophyll concentration. Likewise, similar measured SPAD values were associated with different chlorophyll concentrations for one variety of rice grown under different conditions (RD47, Fig. 5). This implies that estimation of rice leaf chlorophyll concentration from either SPAD value or the response of a camera should factor in rice variety and growth conditions.

These results align with findings that the relationship between SPAD value and chlorophyll concentration varies across species, including birch, wheat, potato (Uddling, 2007), Amazonian trees (Marenco, 2009), rice, maize, soybean, peanut, cotton (Xiong, 2015), and Mexican and Pima cotton (Pauli, 2017).

Structural and textural features of leaves, such as ridges, hairs, and wax coatings, will influence how light interacts with the leaf, and hence the surface spectrophotometry measurements of chlorophyll concentration, but for the rice leaves used in this study the abaxial surface properties were very similar, so such surface properties are unlikely to explain the observed effects of strain and treatment. However, chlorophyll interactions with incident and scattered photons may be affected by the maturity of chloroplasts and or how these are oriented in the leaf with respect to the light path (Howard et al. 2019; Kong and Wada 2014; Wada 2013). Consequently, the complex internal structure of leaves, and the fact that chloroplasts dynamically change position in response to the light environment and growth conditions (Xiong 2015) may explain why we observed different relationships between plants grown under control and nitrogen depleted conditions. For example, nitrogen starvation can reduce chlorophyll a, and accessory pigments such as carotenoids (Cetner et al. 2017) and also the leaf thickness due to reduced number of cell layers (Yamamoto and and Saigusa 2002). Despite making measurements at similar times during the day, this suggests that genetic factors (variety dependence of nitrogen assimilation) as well and nutrient availability (treatment dependence) could both be relevant.

This study made no attempt to investigate causes of these variations, but while estimates of SPAD value from leaf images were relatively insensitive to the specific choice of reference reflectance spectra when using PhotoFolia, the reference spectra used substantially impacted prediction of chlorophyll concentration. Unsurprisingly, a generic linear model for predicting chlorophyll concentration that combined variety- and condition-specific reflectance spectra was ineffective (Fig. 6F).

We note also that although the spectrophotometrically measured chlorophyll concentration should be a ground truth value, solvent-based extractive techniques produce several sources of uncertainty. While every attempt was made to control laboratory factors, such as solvent quality, grinding technique, post sampling storage, extraction time & temperature, chlorophyll is a biologically active molecule, a potentially unstable pigment, which can undergo photo-degeneration during sample

preparation and measurement (Petrović 2017). In addition, the absorption-based measurements recovered from the chlorophyll extract are themselves prone to a variety of uncertainties ranging from spectrometer calibration errors at critical wavelengths to interference from additional pigments (Aminot 2000; Riemann 1978). Also, as in the case of the SPAD value measurements, the chlorophyll extraction was not made from the same identifiable region of the leaf that was photographed when making image-based estimates.

It is difficult to quantify the contributions of these different sources of uncertainty. And despite ostensibly being a ground truth measurement, spectrophotometry is likely to exhibit a greater level of uncertainty compared to the raw measurements recovered from hardware SPAD meters and camera images (Tan 2021), but crucially, assay-based spectrophotometry is much less affected by the way that chlorophyll is distributed in the leaf. So ultimately, it is the appropriate ground truth for quantifying the true chlorophyll content.

Practical utility of image-based leaf chlorophyll measurement

This study shows that image-based measurements of leaf chlorophyll implemented by PhotoFolia has similar performance and limitations to SPAD for informing agricultural practise. This study used several models of mobile phone and was conducted under normal (diffuse) natural daylight illumination and confirmed that PhotoFolia is robust to the choice of camera and variation in illumination. If absolute leaf chlorophyll concentration is desired then a calibration model is required, which for PhotoFolia is provided by the choice of reference reflectance spectra and associated chlorophyll concentration (ground truth) measurements that have previously been acquired. These enable the camera response to be mapped accurately onto chlorophyll concentration (fig. 2). In the case of the SPAD meter, a similar set of measurement pairs (SPAD values and chlorophyll concentrations) are required for calibration. PhotoFolia has the advantage of integration within a phone app, so that the user simply selects the leaf type and condition, and the correct calibration is applied. By comparison, existing solutions such as the Minolta SPAD 502+ meter require separate calibration.

Further, an image-based chlorophyll measurement procedure that can be implemented on widely available mobile phones is obviously convenient. Moreover, mobile phones provide GPS and are often operated on-line, enabling integration of geo-located chlorophyll concentration data with cloud-based analytics.

However, PhotoFolia is not an entirely hardware independent solution, it still requires a colour standard to enable a calibrated camera response. Some care is needed when acquiring images to avoid variations in the spectral distribution of light falling across the colour standard and avoid occlusion or shadowing of the leaf in the colour standard sample aperture. However, the image can be reviewed by both by the user and processed to establish whether criteria required for accurate estimates have been met. Many potential sources of uncertainty in the final chlorophyll concentration estimate can be mitigated. In addition, the use of colour cards is familiar to some potential users.

The main uncertainty at this point is precisely how the information about chlorophyll concentration or indeed, any property of a plant that can be inferred from its spectral reflectance, can contribute towards improved decision making in the field. The fact that PhotoFolia enables chlorophyll concentration estimates from leaf images that are within 10% of a laboratory-based assay almost instantly and at almost no cost suggests potential to impact horticultural practise.

ACKNOWLEDGEMENTS

J. Anderson contributed to the development of analytical and data processing methods, data analysis, software development, and manuscript. K. Boonyaves and H. Okamoto contributed to the experiment design, data collection, and to the writing of this manuscript. N. Karaket, W. Chuekon, and N. Chabang provided valuable support in gathering and organising the experimental data. M. Garvie developed the mobile phone application used in the study. Daniel Osorio and K. Supaibulwatana contributed to the planning the study, experimental design and preparation of the manuscript.

This research was supported by the Mahidol-Sussex seed fund.

REFERENCES

Agarwal, A., Kumar, P., Snehasish, D., and Gupta, D. (2021). Smartphone-assisted real-time estimation of chlorophyll and carotenoid concentrations and ratio using the inverse of red and green digital color features. *Theor. Exp. Plant Physiol., 33*, 293-302

Ali, M.M., Al-Ani, A., Eamus, D., and Tan, D.K.Y. (2012). A New Image Processing Based Technique to Determine Chlorophyll in Plants. *American-Eurasian J. Agric. & Environ. Sci., 12*, 1323-1328

Aminot, A.a.R., F. (2000). Standard procedure for the determination of chlorophyll a by spectroscopic methods. In, *International Council for the Exploration of the Sea*

Cetner, M.D., Kalaji, H.M., Goltsev, V., Aleksandrov, V., Kowalczyk, K., Borucki, W., & Jajoo, A. (2017). Effects of nitrogen-deficiency on efficiency of light-harvesting apparatus in radish. *Plant Physiol Biochem, 119*, 81-92

Chiao, C.C., Osorio, D., Vorobyev, M., & Cronin, T.W. (2000). Characterization of natural illuminants in forests and the use of digital video data to reconstruct illuminant spectra. *J Opt Soc Am A Opt Image Sci Vis, 17*, 1713-1721

Evans, J.R. (1989). Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia, 78*, 9-19

Finlayson, G.D., Hordley, S. and Hubel, P. M. (1998). Recovering Device Sensitivities with Quadratic Programming. In, *The Sixth Color Imaging Conference: Color Science, Systems, and Application* (pp. 90-95)

Gausman, H.W. (1985). Plant Leaf Optical Properties in Visible and Near-Infrared Light. In (p. 76): Texas Tech University

Howard, M.M., Bae, A., & Koniger, M. (2019). The importance of chloroplast movement, nonphotochemical quenching, and electron transport rates in light acclimation and tolerance to high light in Arabidopsis thaliana. *Am J Bot, 106*, 1444-1453

Ibrahim, N.U.A., Abd Aziz, S., Jamaludin, D. and Harith, H.H. (2021). Development of smartphone-based imaging techniques for the estimation of chlorophyll content in lettuce leaves. *Food Research, 5*, 33-38

IRRI (2005). Use of leaf color chart (LCC) for N management in rice. In P.I.R.R. Institute (Ed.)

Jiamtaea, P., Onwimola, D., Chanpraserta, W., Thongketb, T., and Prasertsakc, A. (2017). Varietal verification of Thai rice using ultrathin layer isoelectric focusing of seed proteins. *ScienceAsia, 43*, 145-154

Kong, S.G., & Wada, M. (2014). Recent advances in understanding the molecular mechanism of chloroplast photorelocation movement. *Biochim Biophys Acta, 1837*, 522-530

Kulig, B., Klimek-Kopyra, A., Ślizowska, A., Oleksy, A., Skowera, B., Lepiarczyk, A., & Grygierzec, W. (2024). A comparison of the methods used to assess the nutritional status of selected crop species. . *Journal of Water and Land Development*

Lichtenthaler, H.K. (1987). *Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes*. Elsevier

Markwell, J., Osterman, J.C., & Mitchell, J.L. (1995). Calibration of the Minolta SPAD-502 leaf chlorophyll meter. *Photosynth Res, 46*, 467-472

Monje, O., and Bugbee, B. (1992). IInherent Limitations of Nondestructive Chlorophyll Meters: A Comparison of Two Types of Meters. *Horticultural Science, 27*, 69-71

Peng, S., Garcia, F.V., Laza, R.C., Sanico, A.L., Visperas, R.M., Cassman, K.G. (1996). Increased nitrogen use efficiency using a chlorophyll meter on high-yielding irrigated rice. . *Field Crops Research, 47*, 243-252

Petrović, S., Zvezdanović, J., Marković, D. (2017). Chlorophyll degradation in aqueous mediums induced by light and UV-B irradiation: An UHPLC-ESI-MS study. *Radiation Physics and Chemistry, 141*, 8-16

Riemann, B. (1978). Carotenoid interference in the spectrophotometricdetermination of chlorophyll degradationproducts from natural populations of phytoplankton. *Limnology and Oceanography, 23*

Shi, H., Jacquemoud, S., Jiang J., Zhou, M., Fabre, S., Richardson, A.D., Wang, S., Jiang, X., and Xiao, Z. (2024). The PROLIB leaf radiative transfer model: Simulation of the dorsiventrality of leaves from visible to mid-wave infrared. *Remote Sensing of Environment, 306*

Tan, L., Zhou, L., Zhao, N., He,Y., Qiu, Z. (2021). Development of a low-cost portable device for pixel-wise leaf SPAD estimation and blade-level SPAD distribution visualization using color sensing. *Computers and Electronics in Agriculture, 190*

Tucker, C.J. (1979). Red and photographic infrared linear combinations for monitoring vegetation. *Remote Sensing of Environment, 8*, 127-150

Uddling, J.G.-A., J., Piikki, K., and Pleijel, H. (2007). Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynthesis Research, 91*, 37-46

Wada, M. (2013). Chloroplast movement. *Plant Sci, 210*, 177-182

Xiong, D., Chen, J., Yu, T., Gao, W., Ling, X., Li, Y., Peng, S., & Huang, J. (2015). SPAD-based leaf nitrogen estimation is impacted by environmental factors and crop leaf characteristics. *Scientific Reports, 295*

Xu, K., & Ye, H. (2023). Light scattering in stacked mesophyll cells results in similarity characteristic of solar spectral reflectance and transmittance of natural leaves. *Sci Rep, 13*, 4694

Yamamoto, A., Nakamura,T., Adu-Gyamfi, J. J.,, & and Saigusa, M. (2002). Relationship between chlorophyll content in leaves of sorghum and pigeonpea determined by extraction method and by chlorophyll meter (SPAD-502). *Journal of Plant Nutrition, 25*, 2295-2301

Yoshida, S. (1976). Routine Procedure for Growing Rice Plants in Culture Solution. . In S.Y.D.A.F.J.H.C.K.A. GOMEZ (Ed.), *Laboratory Manual for Physiological Studies of Rice* (pp. 61-66). LOS BAÑOS, LAGUNA, PHILIPPINES International Rice Research Institute

Zimmermann, N.E., Washington-Allen, R.A., Ramsey, R.D., Schaepman, M.E., Mathys, L., Kötz, B., Kneubühlerx, M., and Edwards, T.C. (2007). *Modern Remote Sensing for Environmental Monitoring of Landscape States and Trajectories*.