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Phenotyping flag leaf nitrogen content in rice using a three-band spectral index

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Abstract Accurate, rapid and non-destructive measurements of rice flag leaf nitrogen content (LNC) are crucial for geneticists and breeders. To help design a less expensive, non-destructive, real-time LNC sensor, we developed a Three-Band Difference Ratio (TBDR) spectral index, TBDR (R_{755} , R_{513} , R_{508}). This spectral index could accurately and rapidly estimate rice LNC in a population of chromosome segment substitution lines with small variation in LNC. The model estimating LNC was validated using a leave-one-out

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21 cross-validation technique; the achieved root mean square error was 0.13% and the relative
22 error was 4.74%. In comparison with SPAD-502plus chlorophyll meter readings and
23 commonly used spectral indices, including GreenSeeker- and Crop Circle-based indices,
24 TBDR (R₇₅₅, R₅₁₃, R₅₀₈) produced higher accuracy in LNC estimation.

25 **Keywords:** Oryza sativa; spectral reflectance; SPAD-502plus chlorophyll meter;
26 chromosome segment substitution lines

27

1. Introduction

A major challenge for crop research in the 21st century is how to predict crop performance based on genetic background. Advances in “next generation” DNA sequencing have greatly enhanced the efficiency of genotyping while reducing its costs. Methods for characterizing plant traits (phenotypes), however, have progressed less rapidly over the decades, and constraints in phenotyping capability limit our ability to dissect the genetics of quantitative traits (Houle et al., 2010).

Chromosome segment substitution lines (CSSLs) can be used in genome-wide association studies to detect and fine-map quantitative trait loci (QTLs) and to study the interactions between those QTLs. Several populations of CSSLs have been developed for rice, and many QTLs for traits of agronomic importance have been detected in this way (Takai et al., 2007; Zhou et al., 2009; Zhu et al., 2009). These results have undoubtedly enhanced the understanding of the genetics of complex traits. However, efficient and accurate phenotyping of large populations is challenging for geneticists and breeders (Houle et al., 2010). This is particularly true when differences between CSSLs are relatively small but relevant.

Leaf nitrogen content (LNC) has been identified as a key plant trait determining photosynthetic capacity (Reich et al., 1998). Traditional measurements of LNC rely on destructive, costly and laborious methods (Ecartot et al., 2013). Spectroscopic methods have been used to provide rapid and non-destructive estimates of leaf and canopy nitrogen contents of crops. Table 1 lists the vegetation spectral indices that have been commonly employed in remote sensing of LNC in crops (e.g., Tian et al., 2011; Li et al., 2014; He et al., 2016). In general, most existing spectral indices are based on two- or three-band

combinations because including more bands may cause the problem of overfitting. The normalized difference vegetation index (NDVI) involving a near-infrared (NIR) band and a red band is one of the most widely used spectral indices in agricultural remote sensing (Rouse et al., 1974). Some NDVI-like two-band indices have been used to improve crop nitrogen management, e.g. the normalized pigment chlorophyll index (NPCI) (Peñuelas et al., 1994), the photochemical reflectance index (PRI) (Peñuelas et al., 1995), the green normalized difference vegetation index (Green-NDVI) (Gitelson et al., 1996), the normalised difference red edge index (NDRE) (Barnes et al., 2000), and the Crop Circle (CC) 730_670 (Erdle et al., 2011). Besides these two-band indices, a three-band index of modified normalized difference has been used to monitor leaf nitrogen status, because it effectively described changes in chlorophyll content (Sims and Gamon, 2002; Feng et al., 2008; Jin et al., 2014). Another three-band spectral index including reflectance at 491 nm, 705 nm and 717 nm was suggested as a good indicator of LNC in rice (Tian et al., 2011). However, these existing indices need further testing.

Table 1 Quantitative linear relationships between flag leaf nitrogen content (LNC) and commonly used spectral indices based on all 130 chromosome segment substitution lines (CSSLs) of rice. The measured LNC values of the 130 CSSLs ranged between 2.28% and 3.26%.

Index	Formula	Reference	R^2	SE (%)
<i>2 bands:</i>				

Normalised difference vegetation index (NDVI1)	$(R_{780}-R_{670})/(R_{780}+R_{670})$	Rouse et al. (1974)	0.01	0.21
Normalised difference spectral index 1 (NDVI2)	$(R_{573}-R_{440})/(R_{573}+R_{440})$	Hansen and Schjoerring (2003)	0.18	0.19
Normalised difference spectral index 2 (NDVI3)	$(R_{503}-R_{483})/(R_{503}+R_{483})$	Stroppiana et al. (2009)	0.23	0.19
Ratio vegetation index 1 (RVI1)	R_{780}/R_{670}	Pearson and Miller (1972)	0.01	0.21
Ratio vegetation index 2 (RVI2)	R_{787}/R_{765}	Fava et al. (2009)	0.12	0.20
NIR1/NIR2	R_{780}/R_{740}	Mistele and Schmidhalter (2010)	0.47	0.15
Optimised vegetation index 2 (VIopt2)	R_{760}/R_{730}	Jasper et al. (2009)	0.51	0.15
Zarco-Tejada & Miller (ZTM) index	R_{750}/R_{710}	Zarco-Tejada et al. (2001)	0.50	0.15
Normalised difference red edge index (NDRE)	$(R_{790}-R_{720})/(R_{790}+R_{720})$	Barnes et al. (2000)	0.50	0.15
Red-edge model index (R-M)	$(R_{750}/R_{720})-1$	Gitelson et al. (2005)	0.51	0.15
Green model index (G-M)	$(R_{750}/R_{550})-1$	Gitelson et al. (2005)	0.47	0.15
Green normalized difference vegetation index (Green-NDVI)	$(R_{750}-R_{550})/(R_{750}+R_{550})$	Gitelson et al. (1996)	0.48	0.15
Photochemical reflectance Index (PRI)	$(R_{570}-R_{531})/(R_{570}+R_{531})$	Peñuelas et al. (1995)	0.01	0.21
Normalized difference cloud index (NDCI)	$(R_{762}-R_{527})/(R_{762}+R_{527})$	Marshak et al. (2000)	0.45	0.16
Normalized pigment chlorophyll index (NPCI)	$(R_{430}-R_{680})/(R_{430}+R_{680})$	Peñuelas et al. (1994)	0.00	0.21

Ratio index (RI-1dB)	R_{735}/R_{720}	Gupta et al. (2003)	0.50	0.15
Soil adjusted vegetation index (SAVI)	$1.5 \times (R_{870} - R_{680}) / (R_{870} + R_{680} + 0.5)$	Huete (1988)	0.02	0.21
Crop Circle (CC) 730_670	$(R_{730} - R_{670}) / (R_{730} + R_{670})$	Erdle et al. (2011)	0.02	0.21
Crop Circle (CC) 760_730	$(R_{760} - R_{730}) / (R_{760} + R_{730})$	Barnes et al. (2000)	0.51	0.15
Crop Circle (CC) NDVI	$(R_{760} - R_{670}) / (R_{760} + R_{670})$	Jasper et al. (2009)	0.00	0.21
GreenSeeker (GS) NDVI	$(R_{774} - R_{656}) / (R_{774} + R_{656})$	Erdle et al. (2011)	0.08	0.20
<i>3 bands:</i>				
R705/(R717+R491)	$R_{705} / (R_{717} + R_{491})$	Tian et al. (2011)	0.41	0.16
R434/(R496+R401)	$R_{434} / (R_{496} + R_{401})$	Tian et al. (2011)	0.01	0.21
Chlorophyll absorption in reflectance index (CARI)	$(R_{700} - R_{670}) - 0.2 \times (R_{700} + R_{550})$	Kim et al. (1994)	0.55	0.14
Modified chlorophyll absorption in reflectance index (MCARI)	$[(R_{700} - R_{670}) - 0.2 \times (R_{700} - R_{550})] \times (R_{700} / R_{670})$	Daughtry et al. (2000)	0.54	0.14
Structural independent pigment index (SIPI)	$(R_{800} - R_{445}) / (R_{800} - R_{680})$	Peñuelas et al. (1995)	0.00	0.21
Modified red-edge normalized difference vegetation index (mND705)	$(R_{750} - R_{705}) / (R_{750} + R_{705} - 2 \times R_{445})$	Sims and Gamon (2002)	0.36	0.17
New double difference index (DDn)	$2 \times R_{710} - R_{660} - R_{760}$	Maire et al. (2008)	0.51	0.15
MERIS terrestrial chlorophyll index (MTCI)	$(R_{754} - R_{709}) / (R_{709} - R_{681})$	Dash and Curran (2004)	0.57	0.14
Double-peak nitrogen index (NDDA)	$(R_{680} + R_{756} - 2 \times R_{718}) / (R_{756} - R_{680})$	Feng et al. (2014)	0.55	0.14
<i>4 bands:</i>				
Red edge position (REP)	$700 + 40 \times [(R_{670} + R_{780}) / 2 - R_{700}]$	Guyot et al. (1988)	0.56	0.14

	$/(R_{740}-R_{700})$			
Vogelmann index (VOG-2)	$(R_{734}-R_{747})/(R_{715}+R_{726})$	Zarco-Tejada et al.	0.51	0.15
		(2001)		

R_i denotes the reflectance at wavelength i in nm. SE denotes the standard error of the regression. Coefficient of determination (R^2) and SE can evaluate the spectral indices.

Based on these spectral indices, some apparatuses have been designed and widely applied to make non-destructive real-time diagnoses of crop nitrogen status and guide in-season nitrogen management (Cao et al., 2017). The handheld GreenSeeker (NTech Industries Inc., Ukiah, California, USA) measures canopy reflectance in the red (656 nm) region and the near infrared (NIR; 774 nm) region to calculate the GreenSeeker NDVI readings (Table 1). It has been applied for site-specific and need-based nitrogen management in rice (Bijay-Singhet al., 2011; Ali et al., 2014). Another widely used optical sensor is the Crop Circle sensor (Holland Scientific Inc., Lincoln, Nebraska, USA), which can produce NDVI-like spectral indices (Table 1). It has been applied for non-destructive and real-time nitrogen status assessment in rice (Cao et al., 2013), wheat (Cao et al., 2017) and potato (Giletto and Echeverria, 2016).

In contrast to the sensors of GreenSeeker and Crop Circle that assess reflectance, the SPAD-502plus chlorophyll meter (Minolta Camera Co., Osaka, Japan) measures transmittance at 650 nm and 940 nm to provide a relative measure of leaf chlorophyll content. The SPAD-502plus is designed as a light-weight hand-held chlorophyll meter; it has been employed as a low-cost, rapid, simple, and non-destructive apparatus to diagnose the nitrogen nutrition status of various crops (Yang et al., 2014).

Although these commonly used spectral indices have effectively estimated LNC in previous studies, it is unclear whether they are effective for accurate phenotyping because variations of LNC may be small among CSSLs. Therefore, the objectives of the present study were (1) to evaluate the applicability and reliability of commonly used spectral indices on estimating LNC of rice; (2) to develop a new LNC-sensitive spectral index that can estimate LNC more effectively than the commonly used spectral indices. The new spectral index may help designing a practical and less expensive, non-destructive, real-time LNC meter, and the anticipated outcome would help to provide efficient and accurate phenotyping of large populations for geneticists and breeders.

2. Materials and methods

2.1. Experimental design and crop cultivation

In this study, a wide population consisting of 130 CSSLs was developed, derived from the crossing and back-crossing of two sequenced rice cultivars: 9311, an elite indica cultivar as the recipient and Nipponbare, a japonica cultivar as the donor (Xu et al., 2010). The experiment was conducted at the Experimental Farm of Yangzhou University, Yangzhou, Jiangsu, China (32°24' N, 119° 23'E) in 2016 following a complete randomized block design, with three replications, three rows per plot, 12 hills per row. The seeds were first sown in a paddy field on 12 May 2016. The seedlings were then transplanted to the experimental field on 10 June 2016. Seedlings were transplanted at a hill spacing of 0.25 × 0.16 m with two seedlings per hill. Nitrogen (60 kg ha⁻¹ as urea), phosphor (30 kg ha⁻¹ as single superphosphate), and potassium (40 kg ha⁻¹ as potassium chloride) were applied and

incorporated just before transplanting. Nitrogen as urea was also applied at mid-tillering (60 kg ha⁻¹) and at panicle initiation (25 kg ha⁻¹).

At anthesis, the stage at which the crop makes the switch from vegetative growth to reproductive growth, measurements were carried out and samples were taken. The stage was selected because photosynthesis at anthesis is pivotal to carbohydrate supply during grain filling and may contribute up to 70% of the final yield of rice (Yang et al., 2010). The nitrogen content in leaves is tightly related to photosynthetic capacity (Gu et al., 2012a, b), as nitrogen is one of the main components of photosynthetic enzymes.

2.2. Data acquisition

2.2.1. Measurement of leaf hyperspectral reflectance and SPAD readings

Spectral measurements were conducted on five dates (9, 11, 16, 22 and 25 August 2016), because CSSLs varied in date of anthesis. Only the CSSLs that had reached anthesis were sampled. For each CSSL, four flag leaves from four randomly selected plants were spectrally measured in situ using an Analytical Spectral Device (ASD) FieldSpec® 3 Full-Range spectroradiometer (Analytical Spectral Devices, Inc., Boulder, CO, USA). The spectral range of the spectroradiometer was 350 nm–2500 nm and its spectral resolution varied from 3 nm for the 350 nm–1000 nm region to 10 nm for the 1000 nm–2500 nm region. The sampling interval was 1.4 nm for the 350–1000 nm region and 2 nm for the 1000–2500 nm region. The spectrum was obtained by interpolation in 1-nm steps.

Spectral measurements were conducted on the leaf adaxial surface using the ASD spectroradiometer coupled via the fibre optic cable to a leaf clip with an internal calibrated light source (Analytical Spectral Devices, Boulder, CO, USA). The leaf clip had two panels, a white panel used for instrument calibration and a black panel used for measurements. The

widths of measured leaves were 1.6 - 2.4 cm, wide enough to fully cover the leaf slip's light spot. In addition, a black circular gasket of 2.2 cm inner diameter was pasted to the leaf clip to avoid the effects of external light through the edges. Reflectance values were recorded at two fifths of leaf length of the four flag leaves, and then averaged to determine a single spectral curve for each CSSL. Then, the moving average of three datum points, equally spaced on either side of a central datum point, was calculated to smooth out the noises of the spectral curves. Only the wavelength range between 400 and 1000 nm was considered to determine leaf reflectance in this study.

SPAD readings were recorded using a SPAD-502plus meter at two fifths of leaf length of the same four flag leaves, and then averaged for each CSSL. For the same CSSL, spectral measurements and SPAD measurements were conducted on the same date.

2.2.2. Determination of leaf nitrogen concentration

Leaf samples for leaf weight and LNC assessments were separated from stems and stored in small paper bags immediately after leaf spectral measurement in field. Samples were oven-dried at 105 °C for two hours, followed by oven-drying at 75 °C until constant weight was achieved. After being weighed, dried leaf samples were ground and passed through a 1-mm screen, and stored in plastic bags for subsequent analysis. The samples were digested and LNC was measured by micro-Kjeldahl analysis with a Kjeldahl apparatus (Kjeltec 8400, FOSS, Sweden). The measured LNC values of the 130 samples (one sample from each CSSL) ranged between 2.28% and 3.26%.

2.3. Data analysis

2.3.1. Determination of optimum spectral index of LNC

Besides the reflectance of single wavelength between 400 and 1000 nm as well as 32 commonly used spectral indices (Table 1), three types of two-band spectral indices and two types of three-band spectral indices were constructed and evaluated. This study only constructed two-band and three-band spectral indices to avoid the problem of overfitting.

The three types of two-band indices included simple difference (SD) indices, simple ratio (SR) indices and normalized difference (ND) indices calculated as follows.

$$SD(R_{\lambda_1}, R_{\lambda_2}) = R_{\lambda_1} - R_{\lambda_2} \quad (1)$$

$$SR(R_{\lambda_1}, R_{\lambda_2}) = \frac{R_{\lambda_1}}{R_{\lambda_2}} \quad (2)$$

$$ND(R_{\lambda_1}, R_{\lambda_2}) = \frac{R_{\lambda_1} - R_{\lambda_2}}{R_{\lambda_1} + R_{\lambda_2}} \quad (3)$$

In Equations 1-3, λ_1 and λ_2 ($\lambda_1 > \lambda_2$) are all possible two-band combinations in the 400–1000 nm region; R_{λ_1} and R_{λ_2} are the reflectance values at λ_1 and λ_2 , respectively.

The contour map method can help efficiently determine the optimum band combinations (Inoue et al., 2012; Jin et al., 2016). This study used contour maps of the values of coefficient of determination (R^2) of the regression between LNC and spectral indices (i.e. SD, SR and ND, respectively) to show and compare the LNC estimation abilities of combinations of two single wavelengths.

This study also constructed and calculated two types of three-band spectral indices including three-band difference ratio (TBDR) indices and three-band sum ratio (TBSR) indices as follows:

$$TBDR(R_{\lambda_1}, R_{\lambda_2}, R_{\lambda_3}) = \frac{R_{\lambda_1}}{R_{\lambda_2} - R_{\lambda_3}} \quad (4)$$

$$TBSR(R_{\lambda_1}, R_{\lambda_2}, R_{\lambda_3}) = \frac{R_{\lambda_1}}{R_{\lambda_2} + R_{\lambda_3}} \quad (5)$$

In Equations 4-5, λ_1 , λ_2 and λ_3 ($\lambda_2 > \lambda_3$) are all possible three-band combinations in the 400–1000 nm region; R_{λ_1} , R_{λ_2} and R_{λ_3} are the reflectance values at λ_1 , λ_2 and λ_3 , respectively.

Using all 130 samples, linear correlation analyses were conducted between LNC and the spectral indices to identify sensitive spectral indices related to LNC using an in-house developed computer program of MATLAB R2014a software (The MathWorks, Inc., Natick, MA, USA). R^2 and standard error (SE) were used to evaluate the spectral indices and determine the optimum LNC-sensitive spectral index.

2.3.2. Model establishment and validation

A linear relationship was established between the determined optimum LNC-sensitive spectral index and the LNC values of rice. A leave-one-out cross-validation (LOOCV) technique was applied to perform an unbiased assessment of the estimation capacity of the model (Bouvier et al., 2015). The model goodness-of-fit was assessed by comparing the root mean square error (RMSE) and relative error (RE) obtained from the LOOCV procedure.

RMSE and RE were calculated as follows:

$$RMSE = \sqrt{\frac{1}{n} \times \sum_{i=1}^n (E_i - M_i)^2} \quad (6)$$

$$RE = \sqrt{\frac{1}{n} \times \sum_{i=1}^n \left(\frac{E_i - M_i}{M_i} \right)^2} \times 100\% \quad (7)$$

where E_i and M_i are estimated and measured values, respectively, and n is number of samples. The estimation is considered excellent if RE <10%, good if 10–20%, fair if 20–30%, and poor if >30% (Jamieson et al., 1991; Zhu et al., 2006).

The final model was obtained by averaging the model coefficients computed at each step of the cross-validation performed (Bouvier et al., 2015).

3. Results

3.1. Relationship between LNC and SPAD readings

The SPAD values of rice flag leaves showed a significant linear relationship with LNC, with an R^2 value of 0.38 and an SE value of 0.17% (Figure 1).

Figure 1. Relationship between leaf nitrogen content (LNC) and SPAD readings of 130 chromosome segment substitution lines.

3.2. Relationships between LNC and reflectance of single wavelength

Linear relationships between LNC and single reflectance were assessed by their correlation coefficient (r) (Figure 2). The values of r varied notably with wavelength, and there were two peaks of absolute values of r located in the green and red edge regions, respectively. Specifically, the absolute values of r were higher than 0.60 only within the wavelength ranges of 519–605 nm and 696–727 nm. LNC showed significant negative relationship with single reflectance within the two wavelength ranges. The highest absolute value of r (i.e. 0.70) appeared at 707 nm, with an SE of 0.15%.

Figure 2. Linear correlation (r) between leaf nitrogen content (LNC) and reflectance of single wavelength.

3.3. Relationships between LNC and commonly used spectral indices

The linear relationships between LNC and 32 commonly used spectral indices were studied, and their LNC estimation abilities were evaluated by R^2 and SE (Table 1). Among

the 32 indices, 13 indices achieved higher R^2 and the same or lower SE than the reflectance at 707 nm did. In particular, the MERIS terrestrial chlorophyll index (MTCI) performed the best, achieving the highest R^2 of 0.57 and the lowest SE of 0.14%.

3.4. Relationships between LNC and newly proposed spectral indices

Contour maps showed distributions of the R^2 for the linear relationship between LNC and two-band spectral indices (i.e., SD, SR and ND indices, respectively) on two axes of wavelengths in the range of 400–1000 nm (Figure 3). The R^2 values varied dramatically with different wavelength combinations for each type of indices. The regions with high R^2 values indicated effective wavelength combinations for LNC estimation.

For SD indices, the contours of the matrices presented stronger relationships with LNC in the visible region and red edge region than in the NIR region (Figure 3a). SD indices with R^2 values higher than 0.50 occurred mainly in four distinct regions ($\lambda_1=500\text{--}585$ nm, $\lambda_2=430\text{--}530$ nm; $\lambda_1=610\text{--}680$ nm, $\lambda_2=555\text{--}645$ nm; $\lambda_1=690\text{--}720$ nm, $\lambda_2=590\text{--}700$ nm; $\lambda_1=700\text{--}715$ nm, $\lambda_2=480\text{--}510$ nm).

SR indices and ND indices had similar contour maps (Figures 3b and 3c). In contrast to SD indices, SR and ND indices with R^2 values higher than 0.50 generally occurred in two distinct regions ($\lambda_1=725\text{--}905$ nm, $\lambda_2=710\text{--}745$ nm; $\lambda_1=950\text{--}965$ nm, $\lambda_2=715\text{--}720$ nm).

Figure 3. Contour maps showing the coefficient of determination (R^2) for the linear relationships between rice flag leaf nitrogen content (LNC) and simple difference (SD) indices (a), simple ratio (SR) indices (b), and normalized difference (ND) indices (c), calculated from all possible two-band combinations in the range of 400–1000 nm using all 130 chromosome segment substitution lines.

Five spectral indices, i.e., SD (R_{513} , R_{508}), SR (R_{743} , R_{740}), ND (R_{743} , R_{740}), TBDR (R_{755} , R_{513} , R_{508}) and TBSR (R_{707} , R_{716} , R_{668}), were correlated with LNC using all 130 chromosome segment substitution lines (Figure 4), because they were the ones with the

highest R^2 value within their own type of indices. In general, all five indices were significantly correlated with LNC, with R^2 values between 0.53 and 0.64 and SE values between 0.13 and 0.14%, suggesting that they all were sensitive to LNC. Among them, the three-band index TBDR (R_{755} , R_{513} , R_{508}) proved to be superior, achieving the highest R^2 value of 0.64 and the lowest SE value of 0.13%.

Figure 4. Relationships between leaf nitrogen content (LNC) and SD (R₅₁₃, R₅₀₈), SR (R₇₄₃, R₇₄₀), ND (R₇₄₃, R₇₄₀), TBDR (R₇₅₅, R₅₁₃, R₅₀₈) and TBSR (R₇₀₇, R₇₁₆, R₆₆₈), respectively.

All 130 chromosome segment substitution lines were used.

3.5. Identifying and evaluating best spectral indices

Comparing the SPAD value, the reflectance at 707 nm, the 32 commonly used spectral indices (Table 1) and the newly proposed two- and three-band spectral indices (Figure 4), the three-band index TBDR (R₇₅₅, R₅₁₃, R₅₀₈) performed best in estimating LNC of rice, achieving the highest R^2 and the lowest SE. Therefore, it was selected as the optimum remote-sensing indicator of rice LNC.

Using LOOCV, a linear relationship was established between LNC and TBDR (R₇₅₅, R₅₁₃, R₅₀₈) as follows:

$$\text{LNC} = 0.05279 \times \text{TBDR} (R_{755}, R_{513}, R_{508}) + 0.94746 \quad (8)$$

where LNC is in percent, TBDR (R₇₅₅, R₅₁₃, R₅₀₈) is unitless, $R^2 = 0.64$, $P < 0.001$ and $n = 130$. The achieved RMSE and RE were 0.13% and 4.74%, respectively. In the scatter plots of measured LNC against LNC estimated from TBDR (R₇₅₅, R₅₁₃, R₅₀₈) (Figure 5), the data points were close to the 1:1 line. This proved the ability of TBDR (R₇₅₅, R₅₁₃, R₅₀₈) in estimating LNC.

Figure 5. Comparison between the measured leaf nitrogen content (LNC) and the LNC estimated from the leave-one-out cross-validation (LOOCV) using all 130 chromosome segment substitution lines. LNC values were calculated from TBDR (R_{755} , R_{513} , R_{508}). The diagonal lines show the 1:1 relationship.

4. Discussion and conclusions

$SD (R_{\lambda 1}, R_{\lambda 2})$ indices showed strong relationships with LNC within the wavelength range of 500–540 nm (Figure 3a). Among them, $SD (R_{513}, R_{508})$ that represented the spectral reflectance increase within the short wavelength range of 508–513 nm was found to be the best SD index for LNC estimation of rice. TBDR (R_{755} , R_{513} , R_{508}) introduced the

reflectance at 755 nm into SD (R_{513} , R_{508}), and it produced a higher R^2 . Similarly, Liang et al. (2018) developed the first derivative ratio nitrogen vegetation index (FD-SRNI (R_{716} , R_{526})) that was a simple ratio of the first derivative of reflectance at 716 nm to that at 526 nm to estimate the LNC of wheat. Both TBDR (R_{755} , R_{513} , R_{508}) and FD-SRNI (R_{716} , R_{526}) involved the bands from two spectral regions of green and near-infrared, but the former only required three bands while the later required at least four bands.

In comparison to the commonly used spectral indices (Table 1) that were assessed in this study, the new three-band spectral index TBDR (R_{755} , R_{513} , R_{508}) achieved a much higher R^2 value (0.64) and a lower SE value (0.13%). Hence, a larger proportion of the variance in LNC was accounted for by TBDR (R_{755} , R_{513} , R_{508}) with a smaller estimation error.

TBDR (R_{755} , R_{513} , R_{508}) also performed better than the GreenSeeker (GS) sensor, the Crop Circle (CC) sensor and the SPAD-502plus, three apparatuses widely used for non-destructive, real-time diagnosis of crop nitrogen status and guiding in-season nitrogen management (Yang et al., 2014; Cao et al., 2017). Even CC 760_730, the best one of the four GS-based or CC-based spectral indices, only achieved an R^2 value of 0.54 and an SE value of 0.14% (Table 1). The SPAD readings performed even worse than CC 760_730 with an R^2 value of 0.38 and an SE value of 0.17%.

The estimation model based on the best spectral index identified, i.e. TBDR (R_{755} , R_{513} , R_{508}), was validated using a leave-one-out cross-validation (LOOCV) technique, which achieved an RMSE of 0.13% and an RE of 4.74%, respectively. TBDR (R_{755} , R_{513} , R_{508}) only included three bands. It is helpful for designing a future practical and less expensive non-destructive real-time LNC meter. The anticipated outcome would help provide

efficient and accurate phenotyping of large population for geneticists and breeders as the measured LNC variation of the 130 CSSLs was only 0.98%, much smaller than those reported in previous studies, e.g., about 3.70% (Xue et al., 2004), 4.00% (Zhu et al., 2007), and 2.80% (Tian et al., 2011). The future sensor may perform better than some commonly used sensors such as GreenSeeker, Crop Circle and SPAD-502plus.

This study focused on the anthesis stage. Future research will investigate if the spectral index TBDR (R_{755} , R_{513} , R_{508}) also works well at other growth stages. Furthermore, future research will involve more rice cultivars grown at multiple sites.

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