Evaluation of water-use efficiency in foxtail millet (*Setaria italica*) using visible-near infrared and thermal spectral sensing techniques

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Abstract

Water limitations decrease stomatal conductance (g_s) and, in turn, photosynthetic rate (A_{net}), resulting in decreased crop productivity. The current techniques for evaluating these physiological responses are limited to leaf-level measures acquired by measuring leaf-level gas exchange. In this regard, proximal sensing techniques can be a useful tool in studying plant biology as they can be used to acquire plant-level measures in a high-throughput manner. However, to confidently utilize the proximal sensing technique for high-throughput physiological monitoring, it is important to assess the relationship between plant physiological parameters and the sensor data. Therefore, in this study, the application of rapid sensing techniques based on thermal imaging and visual-near infrared spectroscopy for assessing water-use efficiency (WUE) in foxtail millet (*Setaria italica* (L.) P. Beauv) was evaluated. The visible-near infrared spectral reflectance (350-2,500 nm) and thermal (7.5-14 μ m) data were collected at regular intervals from well-watered and drought-stressed plants in combination with other leaf physiological parameters (transpiration rate - E , A_{net} , g_s , leaf carbon isotopic signature - $\delta^{13}C_{leaf}$, WUE). Partial least squares regression (PLSR) analysis was used to predict leaf physiological measures based on the spectral data. The PLSR modeling on the hyperspectral data yielded

accurate and precise estimates of leaf E , g_s , $\delta^{13}C_{leaf}$, and WUE with coefficient of determination in a range of 0.85 to 0.91. Additionally, significant differences in average leaf temperatures ($\sim 1^\circ C$) with a thermal camera were observed between well-watered plants and drought-stressed plants. In summary, the visible-near infrared reflectance data, and thermal images can be used as a potential rapid technique for evaluating plant physiological responses such as WUE.

Keywords

Drought; Visible-Near Infrared Spectroscopy; Leaf Temperature; High-Throughput Sensing; Partial Least Square Regression.

1. Introduction

Drought is a serious agricultural concern that reduces productivity and its severity is predicted to increase in future by the climate change models [1-2]. To improve crop productivity in agricultural lands with limited water availability and under drought conditions, plant breeding programs aim to improve drought tolerance and water-use efficiency (WUE), where WUE is defined as an increase in biomass or yield per unit of water transpired [3-5]. Measuring transpiration rate and WUE of large numbers of plants as required in both plant breeding programs and crop management practices is a labor-intensive and expensive process, which limits the capacity for high throughput phenotyping and early detection, respectively. In this regard, the proximal and remote sensing techniques can offer high-throughput sensing for assessing plant responses to stress conditions [6-8].

In plant breeding, the accuracy of molecular marker-based methods strongly depends on the quality of phenotype measurements [9-10]. However, measuring WUE, transpiration rate (E), stomatal conductance (g_s), and net photosynthetic rate (A_{net}) for a large number of plants in a breeding program over the course of the growing season would be impossible. Yet determining these variables is important to understand plant physiological response to drought

conditions. Due to the complexity and time involved in acquiring these physiological measurements, phenotyping for drought resistance presents a significant bottleneck to plant breeding programs [11].

Leaf carbon isotopic signature ($\delta^{13}\text{C}_{\text{leaf}}$) has been successfully used as a proxy for transpiration efficiency in wheat breeding programs in Australia [12-13]. Leaf $\delta^{13}\text{C}$ values represent the quantity of water used per carbon fixed by the plant [14], and provides another integrated measure of WUE and photosynthetic capacity [15-17]. Compared to conducting gas exchange measurements to calculate E , g_s , A_{net} , and WUE, $\delta^{13}\text{C}_{\text{leaf}}$ is a useful alternative to direct measures of WUE. Nonetheless, sample processing and data collection process is laborious and expensive that limits the rapid data acquisition.

The proximal and remote sensing techniques using visible-near infrared (Vis-NIR) reflectance spectroscopy and thermal imaging has a potential for rapid, non-destructive, automated, and continuous monitoring of plant responses to water stress [18-22]. Peñuelas and Filella [23] stated that Vis-NIR reflectance had the potential to be used in diagnosing plant physiological status, such as plant biomass, photosynthetic pigments (leaf chlorophyll concentration and leaf nitrogen status) and assessment of plant stress. Carter and Knapp [24] also found that spectral reflectance of plants can give information about plant health, and spectral parameters can provide information on physiological response to growth conditions and plant adaptations to environmental changes. However, the role of water in leaf reflectance in the visible region was obscured by the plant pigment absorption, but these pigments (chlorophyll and carotenoids) are highly transparent in NIR region [25]. Rapaport et al. [26] found that visible (VIS)-to-shortwave infrared (SWIR) imaging spectrometers were indicative of stress-induced alterations in midday leaf water potential (Ψ_l), g_s , and non-photochemical quenching (NPQ), which are important leaf physiological measurements. These studies indicate that spectral reflectance of plants has the potential to monitor physiological variation. Although these studies indicate the potential of proximal and remote sensing techniques, quite often these studies are

limited to a few vegetation indices, and/or monitoring of one or two physiological parameters. The selection of suitable methods and reflectance indices remains challenging as the plant spectral reflectance values vary based on the vegetation type and environmental conditions [27]. Therefore, in this study, we evaluated the relationship between proximal sensing data (Vis-NIR spectral reflectance and thermal data) in detail and physiological factors associated with WUE such as A_{net} , E , and g_s using *Setaria italica* (L.) P. Beauv. Moreover, Vis-NIR reflectance data may be used to detect $\delta^{13}\text{C}_{\text{leaf}}$ in C_4 grasses, similar to studies conducted on C_3 crops [28-29].

S. italica (foxtail millet) is a domesticated crop species closely related to the model species *S. viridis* (L.) P. Beauv., and both species are used as models for the development and improvement of C_4 grass species for biofuel and food production. *S. italica* is an ideal model plant to study C_4 photosynthesis and drought resistance because of its cross-compatibility with *S. viridis*, small and tractable genome, and relatedness with major crop species such as maize, sorghum and potential biofuel crops such as switchgrass and *Miscanthus* [30-31]. In addition, its sequence is published, and a quantitative trait locus (QTL) analytical pipeline has been developed [32-33]. A high-throughput sensing technique to screen *Setaria* traits associated with drought tolerance such as WUE would enhance the ample genetic resources currently being developed with the recombinant inbred lines described in [30]. Hence, we evaluated the relationship between the plant physiological factors, E and g_s , A_{net} , three estimates of WUE, and $\delta^{13}\text{C}_{\text{leaf}}$, and sensor data acquired using proximal sensing techniques (visible-near infrared spectroscopy and thermal imaging). The principal objective of this study was to develop and evaluate the statistical model to predict the physiological factors based on the sensory data.

2. Materials and Methods

2.1. Growth conditions and gas exchange measurements

Setaria italica (L.) P. Beauv. (accession B-100; [31]) was grown in 7.5 L pots with standard potting mixture (Sunshine LC1, Sun Gro Horticulture, Bellevue, WA, USA) in a controlled environment growth chambers (Enconair Ecological GC-16) with 16 h photoperiod (including a 2 h ramp at dawn and dusk), and maximum photosynthetic photon flux density of 1,000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Day and night temperatures were maintained at 28 and 18 ± 1 °C, respectively. The experiment consisted of three treatments that differed in the volume of water supplied nightly to maintain a gravimetric water content of about 3.25, 0.9, and 0.5 for the well-watered, moderately water-limited, and severely water-limited treatments, respectively (Fig. 1).

Gas exchange parameters (A_{net} , g_s , E) were measured on the youngest, fully expanded leaves from non-shaded tillers between 11 am and 3 pm. These measurements were made four times throughout the experiment (31, 34, 40, and 43 days after germination). Plant material for stable isotope analysis and aboveground biomass were collected at 54 days after germination. $\text{WUE}_{\text{intrinsic}}$ and $\text{WUE}_{\text{instantaneous}}$ were calculated as A_{net} over g_s and A_{net} over E , respectively. Long term water use efficiency ($\text{WUE}_{\text{long term}}$) was calculated as dry total aboveground biomass per volume of water transpired. A detailed description of the plants used in this study, growth conditions, and gas exchange methods can be found in Ellsworth et al. [34].

2.2. Thermal imaging and image processing

Thermal images were acquired using a thermal camera (A655sc, Flir Systems Inc., Boston, MA) with spectral range of 7.5 - 14.0 μm and a resolution of 640 × 480 pixels. The lens focal length is 13.1 mm, with 45° field of view. The thermal camera was placed approximately 1 m away from each plant and a black background was used when the plant images were acquired (Fig. 2a). The region of interest (ROI) on each image was defined showing the youngest mature leaves at the top of the plants (Fig. 2b). Image segmentation was performed to separate the leaves from the background using a fuzzy c-mean clustering method [35] on the acquired raw data of ROI (Fig. 2c). After segmentation, a mask image (binary image with black and white

pixels representing the leaf area in the ROI) was created on the image area covered by the leaves. The mask was then applied to the corresponding image (thermal image), which comprised of temperature data to extract the average leaf temperature of the selected area (ROI).

Between five and seven weeks after planting, thermal images of each plant were taken on day 31, 34, 40, and 43 at 2 pm in the growth chamber environment. Henceforth, the data set representing these dates will be referred to as data sets I, II, III, and IV, respectively. In addition to the thermal data collection from all plants at 2 pm, an additional set of thermal imaging measurements were made to evaluate the changes in leaf temperature at different time periods on day-40 (8 am, 2 pm, and 8 pm). Thermal images were processed as described above.

2.3. Visible-near infrared reflectance spectra acquisition

A spectroradiometer (FieldSpec, ASD Inc., Boulder, CO) and contact fiber optic probe with leaf clip were used for acquiring the leaf reflectance spectra in the visible-near infrared spectral range (350 - 2,500 nm). The spectral resolution of the spectroradiometer was 3 nm at 700 nm and 8 nm at 1,400 and 2,100 nm, with 25° field of view for the attached fiber optics probe. The spectroradiometer was calibrated with a standard white panel embedded in the leaf clip before collecting the corresponding Vis-NIR spectra. The spectral measurements were acquired from the central area of the three youngest fully expanded leaves on each plant. These three spectra from each plant were averaged prior to analysis. The Vis-NIR spectral reflectance data were collected in the growth chamber at approximately 3 pm, after the acquisition of thermal images. A total of four data sets were acquired (data sets I-IV), similar to the thermal images.

2.4. Transpiration rate prediction from leaf reflectance spectra

During data preprocessing, Vis-NIR spectral reflectance data were normalized with Euclidean distance [36] and then binned with 10 nm wavelength intervals. The total number of

spectral reflectance bands was 215 in the range of 350 to 2,500 nm. Partial Least Squares Regression (PLSR), a model used in this study utilized 215 features (Vis-NIR spectral bands) for predicting the physiological parameters.

PLSR is a linear regression model based on statistical method, which generalizes and combines features from principal component analysis and multiple regression. PLSR is a powerful tool for prediction of a set of dependent variables (observations, physiological parameters in this case) from a very large set of independent variables (predictors, Vis-NIR spectral bands in this case) [37]. PLSR is especially useful when predictors are highly collinear or when the number of predictors is far more than the number of observations, and ordinary least-squares regression either produces coefficients with high standard errors or fails completely. PLSR can reduce the number of predictors to a smaller set of uncorrelated components by eliminating some predictors using stepwise methods or principal component regression. By providing suitable number of predictors (components), PLSR can select the specific number of components that explain maximum covariance between predictors and observations. PLSR then performs least squares regression on these selected components, instead of on the original data to achieve low prediction error [38].

PLSR was used to predict the selected physiological parameters from spectral features, with a relatively large number of PLS components at first. Later, diagnostics from the first fit were used to make a choice of a simpler model with fewer components. The number of components (predictors) was chosen such that the components explained 85% of the variance in the predictor variable. Once the number of components was finalized, a final PLS analysis was performed. To validate the model performance, leave-one-out cross-validation was performed. The addition of thermal data as one of the spectral features and as one of the PLS components was investigated to assess the model performance with the thermal data.

A feature selection procedure using stepwise multilinear regression was also conducted to investigate the potential spectral bands, using E and g_s as target variables (predictor variable)

and hyperspectral data as the response variable. The stepwise regression analysis and PLS analysis were carried out in Matlab (2013a, MathWorks Inc., Natick, MA), and PLS Toolbox (Eigenvector Research Inc., Manson, WA).

2.5. Vegetation indices

Plants under stress absorb and reflect specific wave bands, which have been used to determine several vegetation indices (VIs) for remote sensing. For example, the normalized difference vegetation index (NDVI, [39-40]), normalized difference water index (NDWI, [41]), photochemical reflectance index (PRI, [39-40]) and water band index (WI, [42]) are some of the vegetation indices that has been estimated for stress detection. Therefore, in addition the PLSR analyses described above, differences in these VIs between treatments were analyzed with analysis of variance (ANOVA) as stated in the following section.

2.6. Statistical analysis

All statistics were conducted in R [43]. Shapiro-Wilk Test of normality and Levene's test of homogeneity were conducted and all data were normal and homogeneous [44]. ANOVA using the general linearized model were conducted to determine differences in leaf temperature, E , and g_s across treatments in each data set (I-IV) and to determine differences in leaf temperature at each time point (8 am, 2 pm, and 8 pm) in a single day. To further elucidate the difference in leaf temperature found between well-watered and water-limited plants at times 2 pm and 8 pm, the water-limited treatments were combined and compared to the well-watered treatment using a Welch's t-test. Water band index (WI) across treatments was compared for each dataset using one-way ANOVA.

3. Results and Discussion

3.1. Thermal imaging

Mean leaf temperature of well-watered (WW) plants was significantly lower than that of plants in both water-limited (WL) treatments in data sets I, II and III, and the water-limited treatments combined had a significantly higher leaf temperature than the well-watered treatment (Table 1, Fig. 3). However, leaf temperature did not significantly differ between moderately and severely water-limited plants in any data set (Fig. 3, Table 1). Following the same trend, plants in the well-watered treatment had higher E and g_s than either of the water-limited treatments, and the water-limited treatments were not significantly different from each other (Fig. 3). A possible reason for not observing significant leaf temperature difference in data set IV (day-43) could be that the taller well-watered plants were closer to the lights and may have had higher temperatures caused by heat produced from the lights.

Thermal images made at 8 am, 2 pm, and 8 pm showed different temperature regimes with differences between WW and WL plants throughout the day. Leaf temperature was lowest in the morning (lights reach full strength at 8 am after a two-hour ramp), yet the chamber temperature had already reached the daytime set point of 28°C. The plants were watered nightly at 10 pm, so water availability was expected to be highest during the morning. Therefore, the water-limited plants at 8 am were not experiencing stomatal closure and reduction in transpiration rates compared to the well-watered plants. However, as the plants depleted the finite volume of water available to them, water limitation became more acute in the water-limited treatments as was seen from difference in transpiration rates (measured at 2 pm) and leaf temperature across treatments at 2 and 8 pm. At solar noon (between 11 am and 2 pm), leaf temperature was highest across treatments and water-limited leaves were warmer than leaves of well-watered plants (simulated in the growth chamber; [45]). Although the leaf temperature in all treatments decreased at 8 pm, the difference in leaf temperature between well-watered and water-limited treatments remained. This is because leaf temperature is a function of light intensity and

transpiration among other factors [46]. At 8 pm (when the light intensity started to ramp down), the plants were exposed to lower light intensity, which caused a reduction in leaf temperature in all treatments. Additionally, the WL plants experienced their lowest point of water availability.

Leaf temperature, being inversely proportion to transpiration rate, was highest in the WL plants, which had lower transpiration rates. Even with fluctuating leaf temperature, the treatment effect on leaf temperature remained, indicating that differences in transpiration rate can be detected even when leaf temperature is fluctuating with ambient temperature. Unlike gas exchange measurements that are not practical on a large scale, thermal imaging can readily be used to screen large numbers of plants serving as a substitute technique to monitor relative shifts in transpiration rates in response to a stress such as water limitation. Although field applications present variable conditions affecting leaf temperature, leaf temperature has been correlated with yield in wheat [13, 47-48] and grapes [49]. This correlation between leaf temperature and yield was due to leaf temperature being negatively correlated with g_s .

3.2. Hyperspectral sensing

3.2.1. Model development using individual datasets

Initially, each data set (I-IV) of hyperspectral data was analyzed separately to eliminate the data collection and phenotypic differences (morphology, plant height) among collection days. For each data set, the PLSR analysis was used to predict the physiological measures (E , g_s , $\delta^{13}C_{leaf}$, and leaf- and plant-level measures of WUE) based on visible-near infrared leaf reflectance spectra. High model performances were achieved across all four data sets (Tables 2 and 3; Fig. 5). The R^2 ranged from 0.85-0.91, with low root mean squared error ($RMSE$) for all parameters except $WUE_{intrinsic}$. The probable reason for higher $RMSE$ during $WUE_{intrinsic}$ prediction could be the variability of $WUE_{intrinsic}$ within a treatment, in spite of observing significant differences between the treatments. The $RMSE$ was lower for predicting E and g_s compared with other physiological factors, such as A_{net} , $WUE_{long\ term}$, and $WUE_{instantaneous}$.

The visible-near infrared spectroscopy and thermal imaging represent two independent sensing schemes (imaging/non-imaging) and measurement scales (leaf/canopy). However, in an attempt to understand if canopy-level temperature measures can provide additional understanding on plant physiological characteristics, we integrated the canopy temperature datum with Vis-NIR spectral reflectance data. Our hypothesis was that if plant temperature measures represented physiological conditions that were not represented by Vis-NIR reflectance data, the integration would improve model prediction performances. To investigate this, the normalized temperature data was integrated with the Vis-NIR data (as one of the spectral data features) before adopting PLSR for predicting E and g_s . However, leaf temperature did not improve the performance of the PLS model further (Table 2). The possible reason for this result could be that the Vis-NIR spectral bands included water sensitive bands that are affected by E or g_s [50-51], where thermal data did not provide additional information to improve model performance. Another possible reason could be that the spectral reflectance and physiological measurements were taken at the leaf level, while leaf temperature from thermal images was measured over a large leaf area, being the mean temperature of four leaves. Similarly, incorporating normalized temperature data as one of the PLS components also did not improve the performance of the PLSR model further (data not shown). The limitation of data integration could be different sensing schemes and measurement scales as indicated above. Future consideration can include canopy-level Vis-NIR reflectance images integrated with thermal images to compensate the current limitations.

3.2.2. Model development using combined dataset

As a second approach (without thermal data), the four data sets (I-IV) were combined and tested with PLSR model. The performance of PLSR model was poor with low R^2 and $RMSE$ (data not shown). Each individual plant was measured for the same physiological parameters over all four data sets. The physiological measurements for each plant made in all data sets

were considered as replicates and were averaged if the values fell within the 95 % confidence interval of the mean. Similarly, Vis-NIR spectra across four datasets for each plant were also averaged. The PLSR model was tested on this combined, averaged dataset (Table 4). The PLS model was found to be robust in predicting the physiological factors, with R^2 ranging from 0.87-0.89, with $RMSE$ for all parameters except $WUE_{intrinsic}$ as was found in the analysis of individual dataset.

High-throughput phenotyping requires rapid and accurate measurements of traits of interest such as WUE , E , g_s , and $\delta^{13}C_{leaf}$. The PLSR analysis with cross-validation showed that measurements important to phenotyping and crop management such as A_{net} , g_s , measures of WUE , and $\delta^{13}C_{leaf}$ had similar coefficients of determination (Tables 3 and 4). In general, visible-near infrared spectral measures of WUE at the leaf-level provided a moderately better fit than the whole plant measure ($WUE_{long term}$), indicating that the differences in spectral reflectance may be more sensitive to leaf-level changes than plant-level changes in terms of growth and water use. Considering the difficulty of measuring leaf and plant-level WUE directly, using Vis-NIR spectra provides a promising method to rapidly screen plants for WUE . Accurate measures of E and g_s are important in both plant breeding for WUE and crop management. Using Vis-NIR reflectance spectra to monitor transpiration can also be used for early detection of water stress and to characterize fields based on heterogeneity in soil water availability [52].

3.3. Stepwise regression analysis and vegetation indices

Preliminary studies using stepwise multilinear regression analysis was performed on the combined, averaged data set to evaluate the critical bands potentially contributing to the prediction of E and g_s . Both data analyses resulted in similar bands (Table 5), which could be because of the high correlation ($r = 0.96$) between E and g_s . Some of the bands selected by stepwise multilinear regression analysis have been found to be associated to leaf water content and broadband greenness indices as indicated in Table 5. The reflectance in visible bands such

as 425 nm represents carotenoids [38]. Some bands such as 795 nm are used to estimate NDVI because at these bands the highest reflectance of chlorophyll can be observed over a wide range of conditions [53]. Similarly, reflectance at 819 nm is unaffected by leaf water content. However, reflectance at 1,595 nm can be sensitive to changing leaf water content, which could indicate water stress or variable leaf water content [54-56]. In general, 1,400 nm to 2,500 nm, a region in the near infrared spectra, is more sensitive to leaf water content or water characteristics than the region, 700-1,300 nm [23, 58]. A reflectance index incorporating 760-900 nm and 1,550-1,750 nm has been found to be highly correlated to leaf relative water content ($R^2 = 0.87$) [59]. Others indicate reflectance at bands from 950-970 nm can indicate plant water status [60]. Nevertheless, a thorough crop-specific evaluation of plant physiology and visible-near infrared spectral reflectance is required for establishing strong relationship between spectral band reflectance and physiological response.

Preliminary evaluation using the selected features described in Table 5 (extracted from g_s data) for PLSR model development indicated R^2 values of 0.90 and 0.89 ($RMSE$ of 0.11 and 0.02) for predicting E and g_s using 11 components for each. The range of E and g_s across three water treatments was 1.26-2.41 and 0.09-0.19 mmol H₂O m⁻²s⁻¹, respectively (Fig. 6). A wider range in E and g_s and/or multiple crops are needed to perform spectral feature selection to predict E and g_s , which would improve assessments of WUE in plants.

In addition to the spectral feature selection above, several spectral indices were evaluated to determine whether they represented differences in WUE between treatments, especially given the pre-symptomatic conditions. These findings may be directly and immediately applied to field studies, as they utilize generic spectral bands found in literature. This analysis would evaluate the applicability of vegetation indices from literature [39-42] and identify a potential index that can be used to identify water stress in early stages. The vegetation indices NDVI, NDWI and PRI were not significantly different between the treatments. It should be noted that the plants did not exhibit any wilting symptoms (Fig. 1) at the end of the experiment.

Interestingly, water band index (WI) indicated a significant difference between the treatments in the combined, averaged data set (Table 6). WI is the ratio of reflectance at 900 and 970 nm water bands. As the water content in the leaves increases, the absorption at 970 nm increases with respect to 900 nm. Evaluating individual datasets, it was found that WI was sensitive between treatments in day-43 (data set IV) at later stages of water stress.

4. Conclusions

This study demonstrates a potential use of thermal imaging and visible-near infrared spectroscopy for rapid evaluation of water-use efficiency (WUE) of foxtail millet (*S. italica*) under growth chamber conditions. The PLSR models yielded accurate and precise estimates of E , g_s , leaf and plant-level WUE, and $\delta^{13}C_{leaf}$, when entire spectra were taken into account. These findings highlight a promising approach for developing high-throughput sensing methods to characterize plant water loss, $\delta^{13}C_{leaf}$, and WUE in *S. italica*. Considering the importance of WUE in plant breeding programs, being able to rapidly assess WUE would help alleviate the current phenotyping bottleneck. Similar to $\delta^{13}C_{leaf}$ used to measure transpiration efficiency, remote sensing using PLSR can be used as a quick screening technique to distinguish the extreme genotypes, which can be followed by more precise measurements to tease apart similar phenotypes, if necessary.

Thus, high-throughput sensing using Vis-NIR spectroscopy and thermal imaging allows rapid assessment of phenotypes unlike more laborious methods such as gas exchange measurements. Our future studies will involve model development using selective spectral reflectance bands to evaluate WUE in multiple crops using PLSR model. In addition to hyperspectral imaging, these selective spectral reflectance bands can then be applied to field studies utilizing customized multispectral bands (with desired visible and near infrared filters) for assessing WUE. Our previous research [61-63] has utilized a specialized camera with visible-near infrared bands to evaluate the disease status and crop emergence in field conditions. One

of the challenges in field applications of visible-near infrared sensing is the influence on ambient light condition changes on spectral reflectance values. However, these changes in light intensities can be compensated utilizing standard reference calibration panels during sensory data acquisition. Other techniques such as utilizing light sensors to measure ambient light conditions can also be useful [64]. Similarly, relative differences in thermal data can be utilized to assess WUE [65]. In addition to standard measures such as vegetation indices, specific spectral features to estimate physiological factors such as E , g_s , and leaf and plant-level WUE can be greatly beneficial in understanding the crop physiological responses to the environment.

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Fig. 1. Images of *Setaria italica* at day-54: (a) well-watered, (b) moderately water-limited, and (c) severely water-limited plant. The stressed plants limit their growth as an adaptation to avoid drought.

Fig. 2. Processing of thermal camera images. (a) Raw data, (b) Region of interest extracted from the raw image data, and (c) use of image segmentation to extract a mask (reference image) to estimate the average temperature of leaf area after background subtraction. (a) and (b) are pseudo-color images, with different colors assigned to different temperature ranges for better visualization.

13

14 Fig. 3. Mean leaf temperature (above), transpiration rates (E ; center), and stomatal conductance
15 (g_s ; below) $\pm$ standard error under well-watered, moderately water-limited, and severely water-
16 limited treatments. Transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) and stomatal conductance ($\text{mmol H}_2\text{O}$
17 $\text{m}^{-2} \text{s}^{-1}$) were measured with a LICOR-6400 at $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 28°C , 35 Pa CO_2 , and
18 $\sim 70\%$ RH. Leaf temperature was measured with a thermal camera and is a mean temperature
19 of four leaves. One-way ANOVAs were conducted for each day, so within each data set bars
20 topped with the same letter are not significantly different. In data set IV, leaf temperature was
21 not significantly different across leaf temperatures, but the water-limited treatments together had
22 a higher leaf temperature than the well-watered treatment ($t = 2.7443_{26.488}$, $P = 0.011$).

23

25

26 Fig. 4. The mean leaf temperature at 8 am, 2 pm, and 8 pm on day-40 under well-watered and
 27 moderately and severely water-limited treatments. One-way ANOVAs were conducted for each
 28 time period, so within each data set (e.g. 8 am, 2 pm, or 8 pm) bars topped with the same letter
 29 are not significantly different. Note that the data set collected at 2 pm is the same as data set III
 30 in Fig. 3. As with leaf temperature at 2 pm, both water-limited treatments were combined and
 31 compared to the well-watered treatment, and leaf temperature of water-limited plants was higher
 32 than that of well-watered plants ($t = 2.744_{26.49}$, $P = 0.011$). Like measurements made at 2 pm,
 33 water-limited treatments together were significantly higher than the well-watered treatment ($t =$
 34 $3.478_{28.70}$, $P = 0.002$).

36

37 Fig. 5. The observed versus predicted values using PLS procedure for leaf transpiration rates
 38 ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) for each data sets (I to IV).

39

40 Fig. 6. The observed and predicted values using PLS procedure for leaf transpiration rates (E ;
 41 mmol $\text{H}_2\text{O} \text{m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s ; mmol $\text{H}_2\text{O} \text{m}^{-2} \text{s}^{-1}$), $\delta^{13}\text{C}_{\text{leaf}}$ (‰), and $\text{WUE}_{\text{long_term}}$ (g
 42 of aboveground biomass per kg of water transpired) for combined, averaged data set.

1 Table 1. One-way ANOVAs table comparing leaf temperature, transpiration rate, and stomatal
2 conductance across treatments for each data set. *P* values indicate the statistical significance.
3 In data set III, leaf temperature for both water limited treatments were combined, and a Welch's
4 T-test was performed. The leaf temperature in the water-limited treatment was significantly
5 higher than that of the well-watered treatment ($t = 2.7443_{26.488}$, $P = 0.011$).

Data sets	Transpiration rate		Leaf temperature		Stomatal conductance	
	$F_{ndf, ddf}$	P	$F_{ndf, ddf}$	P	$F_{ndf, ddf}$	P
I	5.897 _{2, 29}	0.0071	7.036 _{2, 30}	0.003	10.11 _{2, 29}	< 0.001
II	7.886 _{2, 24}	0.0023	7.412 _{2, 30}	0.002	9.936 _{2, 24}	< 0.001
III	20.29 _{2, 26}	< 0.0001	3.05 _{2, 30}	0.062*	30.27 _{2, 26}	< 0.0001
IV	9.519 _{2, 22}	0.0011	1.409 _{2, 30}	0.26	16.28 _{2, 22}	< 0.0001

6 * Not significant at $\alpha=0.05$. All other *P* values are statistically significant.

7 Table 2. A comparison of PLSR prediction model parameters using either Vis-NIR spectra and thermal imaging together or Vis-NIR
8 spectra alone. Coefficient of determination (R^2) and corresponding root mean square error ($RMSE$) were calculated for transpiration
9 rate (E ; mmol H₂O m⁻² s⁻¹) and stomatal conductance (g_s ; mmol H₂O m⁻² s⁻¹). In parenthesis, CV represents $RMSE$ during cross-
10 validation.

Data set	PLS Results	PLS Comp	Without Thermal Data			PLS Comp	With Thermal Data		
			E	PLS Comp	g_s		E	PLS Comp	g_s
I	R^2	13	0.86	13	0.90	14	0.88	13	0.87
	RMSE (CV)		0.16 (0.87)		0.01 (0.05)		0.15 (0.92)		0.01 (0.05)
II	R^2	8	0.86	8	0.86	8	0.87	8	0.85
	RMSE (CV)		0.18 (0.56)		0.02 (0.06)		0.18 (0.63)		0.02 (0.07)
III	R^2	10	0.88	10	0.87	10	0.88	11	0.88
	RMSE (CV)		0.16 (0.57)		0.02 (0.06)		0.16 (0.63)		0.02 (0.07)
IV	R^2	11	0.91	10	0.86	11	0.86	11	0.87
	RMSE (CV)		0.13 (0.86)		0.01 (0.06)		0.15 (0.91)		0.01 (0.07)

12 Table 3. PLSR prediction model parameters using Vis-NIR spectral reflectance data. In each dataset (I to IV), coefficient of
 13 determination (R^2) and corresponding root mean square error ($RMSE$) were calculated for photosynthetic rate (A_{net} ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$),
 14 stomatal conductance (g_s ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), $\delta^{13}\text{C}_{leaf}$ (‰), $WUE_{intrinsic}$ (A_{net}/g_s), $WUE_{instantaneous}$ (A_{net}/E), and $WUE_{long term}$ (aboveground
 15 biomass per water transpired). In parenthesis, CV represents $RMSE$ during cross-validation.

Data set	PLS Results	PLS Comp	A_{net}	PLS Comp	$\delta^{13}\text{C}_{leaf}$	PLS Comp	$WUE_{intrinsic}$	PLS Comp	$WUE_{instantaneous}$	PLS Comp	$WUE_{long term}^*$
I	R^2	13	0.88	10	0.88	12	0.88	12	0.85	12	0.88
	RMSE (CV)		0.88 (4.71)		0.17 (0.51)		6.49 (28.33)		0.71 (3.15)		0.40 (1.51)
II	R^2	9	0.87	10	0.87	8	0.86	11	0.89	12	0.86
	RMSE (CV)		1.47 (5.50)		0.18 (0.61)		10.59 (32.48)		0.64 (3.49)		0.43 (2.09)
III	R^2	10	0.88	13	0.87	10	0.86	11	0.87	10	0.85
	RMSE (CV)		0.90 (3.06)		0.18 (1.08)		11.72 (45.51)		0.54 (2.50)		0.44 (1.41)
IV	R^2	10	0.91	12	0.87	11	0.88	11	0.90	13	0.87
	RMSE (CV)		0.77 (3.42)		0.18 (0.69)		6.61 (39.69)		0.56 (3.85)		0.72 (2.27)

Table 4. PLSR prediction model parameters using combined, averaged Vis-NIR spectral reflectance data and physiological factors data. The coefficient of determination (R^2) and corresponding root mean square error ($RMSE$) were calculated for photosynthetic rate (A_{net} ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration rate (E ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), $\delta^{13}\text{C}_{\text{leaf}}$ (‰), $WUE_{\text{intrinsic}}$ (A_{net}/g_s), $WUE_{\text{instantaneous}}$ (A_{net}/E), and $WUE_{\text{long term}}$ (aboveground biomass per water transpired). In parenthesis, CV represents $RMSE$ during cross-validation.

Parameters	PLS Components	R^2	$RMSE$ (CV)
A	10	0.88	0.62 (1.67)
E	11	0.89	0.11 (0.41)
g_s	11	0.87	0.01 (0.04)
$\delta^{13}\text{C}_{\text{leaf}}$	13	0.87	0.18 (0.75)
$WUE_{\text{intrinsic}}$	12	0.88	7.49 (34.60)
$WUE_{\text{instantaneous}}$	11	0.87	0.47 (1.83)
$WUE_{\text{long term}}$	15	0.87	0.41 (2.46)

25 Table 5. Stepwise multilinear regression analysis ($P = 0.15$) to select spectral bands using
 26 combined, averaged data set with stomatal conductance (g_s) and transpiration data (E).

Selected wavelengths (nm)	Details	References
Using g_s : 425, 785, 795, 815, 1365, 1595, 1615, 1625, 1655, 1665, 1815, 2295	795 nm similar to 800 nm used in NDVI, 815 nm, 1595 nm, and 1655 nm similar to 819 nm, 1599 nm, and 1649 nm, respectively used in normalized difference infrared index and moisture stress index.	[53-57]
Using E : 785, 795, 1365, 1645, 1655, 1675, 1835		

28 Table 6. Comparison of water band index (WI) of plant under different water treatments. The
 29 values represent mean WI $\pm$ standard error. Means followed by the same letter are not
 30 significantly different.

Treatment	Combined data	I	II	III	IV
Well-watered	1.033 $\pm$ 0.001 ^a	1.030 $\pm$ 0.002 ^a	1.031 $\pm$ 0.001 ^a	1.036 $\pm$ 0.002 ^a	1.033 $\pm$ 0.002 ^a
Moderately water-limited	1.030 $\pm$ 0.001 ^b	1.029 $\pm$ 0.001 ^a	1.031 $\pm$ 0.001 ^a	1.034 $\pm$ 0.001 ^a	1.023 $\pm$ 0.002 ^b
Severely water-limited	1.029 $\pm$ 0.001 ^b	1.030 $\pm$ 0.002 ^a	1.031 $\pm$ 0.002 ^a	1.032 $\pm$ 0.009 ^a	1.024 $\pm$ 0.002 ^b

Graphical Abstract