Spectral phenotyping of physiological and anatomical leaf traits related with maize water status

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One sentence summary: Hyperspectral phenotyping has the potential to act as a surrogate for standard reference measurements of physiological and anatomical leaf traits related with water stress in maize.

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Abstract (250 words max)

Advancements in phenotyping techniques capable of rapidly and non-destructively detecting impacts of drought on crops are necessary to meet the twenty-first century challenge of food security. Here, we describe the use of hyperspectral reflectance to predict variation in physiological and anatomical leaf traits related with water status under varying water availability in six maize (Zea mays L.) hybrids that differ in yield stability under drought. We also assessed relationships among traits and collections of traits with yield stability. Measurements were collected in both greenhouse and field environments, with plants exposed to different levels of water stress or to natural water availability, respectively. Leaf spectral measurements were paired with a number of physiological and anatomical reference measurements, and predictive spectral models were constructed using a partial least squares regression (PLSR) approach. All traits were relatively well predicted by spectroscopic models, with external validation (i.e., by applying PLSR-coefficients on a dataset distinct from the one used for calibration) goodness-of-fit ($R^2$) ranging from 0.37 to 0.89 and normalized error ranging from 12–21%. Correlations between reference and predicted data were statistically similar for both greenhouse and field data. Our findings highlight the capability of vegetation spectroscopy to rapidly and non-destructively identify a number of foliar functional traits affected by drought that can be used as indicators of plant water status. Although we did not detect trait coordination with yield stability in the hybrids used in the current study, expanding the range of functional traits estimated by hyperspectral data can help improve trait-based breeding approaches.

Key words

Gas-exchange, hyperspectral phenotyping, leaf thickness, partial least-squares regression, reflectance spectroscopy, stomatal density, water status, Zea mays.
INTRODUCTION

One of the more influential, yet unpredictable, factors expected to change in future environments over the next 50 years will be the intensity and frequency of precipitation events (IPCC, 2014). These changes will likely have a dramatic effect on plant metabolism, growth and production (Pryor et al., 2013; Miao et al., 2017). Over a comparable time period, global food demand is predicted to double, requiring intensification of agricultural production systems (Tilman et al., 2011). The rapid development and adoption of climate-resilient crop genotypes capable of maintaining comparable yield production under favorable and stress conditions (i.e., with high yield stability) is important to overcome this twenty-first century challenge (Mickelbart et al., 2015). Advancements in phenotyping techniques capable of rapidly assessing the effects of drought on plant functional responses from a wide genetic range is necessary to understand plant traits required to maintain yield stability under predicted future environmental conditions (Araus et al., 2012; Cotrozzi et al., 2017a; Miao et al., 2017).

Maize (Zea mays L.) represents an excellent species for developing phenotyping approaches. It is one of the most important crops globally for humans and other animals (Miao et al., 2017), and while yield has increased in absolute value in the last decades, mainly due to genetic gain and improved agronomic practices, the negative impact of drought on maize productivity has also increased due to increased frequency and intensity of drought events (Araus et al., 2012; Lobell et al., 2014). Maize also uses more water at specific developmental stages (e.g., during flowering), making the optimal water management of this species challenging (Araus et al., 2012). Moreover, the functional responses that are associated with increased yield stability and targeted by maize breeders, such as the ability to maintain photosynthesis under stressfull conditions, limit transpiration, increase water-use efficiency, and regulate osmotic adjustment (Ribaut et al., 2009) are difficult, time-consuming, and mostly destructive to measure by standard approaches (e.g., those performed by classic infrared gas analysers, Scholander-type pressure chambers and vapor pressure osmometers). All of these aspects make monitoring of a large number of individual plants logistically impractical.

To date, a number of foliar anatomical, physiological, and biochemical traits have been successfully quantified using vegetation spectroscopy (Asner et al., 2011; Couture et al., 2013, 2016; Serbin et al., 2015; Cotrozzi et al., 2017a). The estimation of these traits from leaf reflectance relies on variations in absorption as a consequence of vibrational excitation of molecular bonds, primarily C-H, N-H and O-H bonds at specific wavelengths in the visible (VIS; 400–700 nm), near-infrared (NIR; 700–1100 nm) and short-wave infrared (SWIR; 1100–2400 nm) spectral regions. Model calibrations are accomplished by pairing leaf spectral signatures with independent and reliable reference measurements. Plant traits are modeled as a function of the spectra using multivariate methods, such as partial least squares regression (PLSR; Wold et al., 2001) or least absolute shrinkage and selection operator (Lasso; Tibshirani, 2011). Best practices suggest that these models are validated using independent samples, after which models can be used to predict the variable of interest in unknown samples on the basis of their spectral reflectance alone (Couture and Lindroth, 2012; Schweiger, 2020). Importantly, leaf reflectance measurements are rapid, taking only seconds, non-destructive, and relatively inexpensive. Although the purchase of a spectrometer can range from hundreds to tens of thousands of US dollars, the expenses to run such instruments are minimal compared with the cost and maintainece of other analytical instrumentation (e.g., HPLC). A spectral approach also provides the potential to assess considerably more individual plant traits in situ and in vivo over multiple time periods than standard reference measurements alone (e.g., those performed with classic ecophysiological techniques or wet chemistry). In addition, this approach can help to monitor plant function over large geographic regions if scaled to remote sensing collections from air- or spaceborne platforms (Cotrozzi et al., 2017b).

Although the use of hyperspectral reflectance (by both imaging or non-imaging sensors) is broadly regarded as a promising phenotyping approach in agriculture (Weber et al., 2012; Araus and Cairns, 2014; Serbin et
al., 2015; Heckmann et al., 2017; Yendrek et al., 2017; Couture et al., 2018), the potential of this technique is
still not fully realized. At present, studies aimed at understanding relationships among leaf optical properties
and photosynthetic metabolism (e.g., Doughty et al., 2011; Serbin et al., 2012, 2015; Ainsworth et al., 2014;
Yendrek et al., 2017; Heckmann et al., 2017; Silvia-Perez et al., 2017) have estimated only a relatively small
number of traits, focusing on the maximum rates of ribulose bisphosphate (RuBP) carboxylation (V_{\text{max}}) and
electron transport (J_{\text{max}}). Similarly, while plant water status has been the focus of a number of studies
utilizing foliar optical properties in the last decades (e.g., Hunt et al., 1987; Peñuelas et al., 1993; Gao, 1996;
Ceccato et al., 2002; Sims and Gamon, 2003; Cheng et al., 2008; González-Fernández et al., 2015), these
studies have primarily focused on foliar water content or changes in water content. To date, only a few
studies (Rodríguez-Pérez et al., 2007; Santos and Kaye, 2009; De Bei et al., 2011; Rallo et al., 2014;
Rapaport et al., 2015; Cotrozzi et al., 2017a) have examined the ability of reflectance spectroscopy to
estimate leaf water potential. These studies, however, have been limited to C3 species, and mostly grapevine
(Vitis vinifera), which potentially presents a limitation when dealing with C4 species because of different
leaf morphology, physiology and biochemistry of the two photosynthetic types (Yendrek et al., 2017).
Moreover, none of these studies have examined the ability of spectral measurements to estimate individual
components of leaf water potential (e.g., osmotic potential). Maize represents a model C4 species, and
reflectance spectroscopy has been used to assess the quality of maize leaves (Weber et al., 2012; Heckmann
et al., 2017; Yendrek et al., 2017), seeds (Spielbauer et al., 2009) and forage (Volkers et al., 2003). To date,
however, few studies have explored the capacity of spectroscopy to estimate important fresh leaf traits in
maize plants under drought stress.

In this study, we tested the capability of reflectance spectroscopy to characterize physiological and
anatomical responses of six maize hybrids with different sensitivities to drought, determined by previously
established differences in yield stability. We collected spectral leaf signatures from maize hybrids growing
under both controlled greenhouse conditions and field conditions. Our objectives in this study were to (i)
develop statistical models to estimate leaf gas-exchange, greenness, water status, thickness, and stomatal
density, and (ii) compare the performance of models built using only greenhouse samples with models
including also field samples. Further, we tested relationships among physiological and morphological
responses of maize hybrids and their yield stability under drought conditions.

RESULTS

Prediction of leaf traits

To optimize model performance, we initially examined numerous models containing different wavelength
ranges, characterized by specific absorption features in known literature that are directly or indirectly related
to specific traits (Supplemental Table S1). Final models for gas-exchange traits utilized the wavelength range
500–900 nm for net CO₂ assimilation (A), transpiration (E), stomatal conductance (g_s), intercellular CO₂
concentration (C_i), instantaneous water-use efficiency (WUE_i), intrinsic water-use efficiency (WUE_in) and
leaf temperature (T_l) (Supplemental Table S1). Cross validated models accurately characterized A, E, g_s and
T_l (Table 1; Fig. 1a,b,c,g). Performance metrics of cross validated modes of C_i, WUE_i and WUE_in models
were slightly lower than those of A, E, g_s and T_l (Table 1; Fig. 1d,e,f). Standardized PLSR coefficients and
the variable important to the projection (VIP) measures exhibited similar profiles for both sets of models.
Specifically, VIP highlighted important wavelengths around 550 nm and from 650 to 750 nm (Fig. 2a-g).
While statistically significant (r = 0.52, P >0.001, n = 61), the correlation between WUE_in calculated using
either observed or predicted values of A and g_s was lower and the bias higher than estimation accuracy of
modelling WUE_in directly (Fig. 2f).

The most optimal leaf chlorophyll concentration (Chl_{SPAD}) PLSR model used the 600–900 nm spectral
region, and cross-validated models estimated chlorophyll concentration well (Supplemental Table S1; Table
Similar to gas-exchange traits, ChlSPAD standardized coefficients and VIP values were most pronounced in the 650–750 nm spectral region (Figure 2h).

The best model performance for leaf water potential ($\Psi_w$) was found using the spectral full-range (400–2400 nm), whereas best model performance for leaf osmotic potential ($\Psi_z$) and leaf osmotic potential at full turgor ($\Psi_{z100}$) utilized the wavelength range 1400–2400 nm. Cross-validated models predicting $\Psi_w$, $\Psi_z$ and $\Psi_{z100}$ preformed reasonably (Table 1; Fig. 1i,j,k). Standardized coefficients and VIP profiles for $\Psi_w$ were relatively flat, showing some peaks around 600, 1500 and 1900 nm, whereas $\Psi_z$ and $\Psi_{z100}$ were more variable, with important wavelengths around 1900 nm (Fig. 2i,j,k). Final models for relative water content (RWC) and succulence (Suc) utilized the 950–2400 nm region (Supplemental Table S1). Predictive models accurately characterized RWC and Suc (Table 1; Fig. 1l,m). Both RWC and Suc standardized coefficients and VIP values were most pronounced in the 1350–1500 nm and 1900–2100 nm spectral regions (Fig. 2l,m). There was no relationship between the the estimation of $\Psi_{z100}$ calculated using either observed or predicted values of $\Psi_z$ and RWC ($r = 0.11$, $P = 0.40$, n = 61). No field measurements were collected for $\Psi_{z100}$, RWC or Suc; therefore, models were developed only from greenhouse-collected data.

Specific leaf area (SLA) was accurately predicted with a PLSR model that utilized wavelengths from 1400 to 2400 nm (Supplemental Table S1). Cross-validated models estimated SLA very accurately (Table 1; Fig. 1n). SLA standardized coefficients and VIP values highlighted important wavelengths around 1500 nm, from 1850 to 2050 nm, and around 2280 nm (Fig. 2n).

The best performance for the total (sum of abaxial and adaxial surfaces) stomatal density (TSD) model was found using the wavelength range 1400–1800 nm (Supplemental Table S1) and cross-validated models yielded sub-optimal estimations (Table 1, Figure 1o). TSD standardized coefficients and VIP values were highly variable throughout the whole spectral-range used (Figure 2o). No field measurements were collected for TSD; therefore, models consisted only of data from the greenhouse. Best models for abaxial and adaxial stomatal density were less accurate than TSD (average $R^2$: 0.17 and 0.10, respectively, using the 1400–1800 nm range).

Averaged fit statistics ($R^2$, RMSE, bias, NRMSE) for external validations were similar to those registered for cross-validation for A, E, $g_s$, Ci, WUEi, WUEin, Tl, Suc, SLA and TSD, whereas they were slightly lower for ChlSPAD ($R^2$: 0.61 vs 0.44 for cross- and external validation, respectively), $\Psi_w$ ($R^2$: 0.63 vs 0.40), $\Psi_z$ ($R^2$: 0.60 vs 0.34), $\Psi_{z100}$ ($R^2$: 0.53 vs 0.40) and RWC ($R^2$: 0.90 vs 0.65, Table 2).

Using A, $\Psi_w$, $\Psi_z$ and SLA as testing traits, the performances of models built that included field and greenhouse data or only greenhouse data were comparable for calibration and cross-validation (Supplemental Table S1). For external validation, the performance of the models built using both greenhouse and field samples was similar whether validations were performed using a dataset containing both greenhouse and field samples (as reported above, Table 2) or on only field samples, with the exception of $A$ ($R^2$: $A$, 0.86 vs 0.31; $\Psi_w$, 0.40 vs 0.41; $\Psi_z$, 0.34 vs 0.34; SLA, 0.77 vs 0.69). However, predictions generated from a data set containing only field samples were dramatically compromised if using coefficients from models built from only greenhouse data, with the exception of $\Psi_z$, which was slightly stronger ($R^2$: $A$, 0.31 vs 0.00; $\Psi_w$, 0.41 vs 0.07; $\Psi_z$, 0.34 vs 0.46; SLA, 0.69 vs 0.35).

For all leaf traits, modelling performance of Lasso using the full spectral range (400–2400 nm) was lower or comparable to PLSR. Using A, $g_s$, Ci and $\Psi_z$ as testing traits, the Lasso modelling performance decreased when using the smaller spectral regions used in the final PLSR modelling (Supplemental Table S2).

**Correlations among leaf traits and leaf traits with spectral indices**

Similar correlations were found either using only greenhouse samples or both greenhouse and field samples together (Supplemental Tables 3 and 4). All significant and strong correlations ($P < 0.05$, $r > 0.4$) among leaf traits of greenhouse samples using reference values were confirmed by using the predicted values, except
RWC with $T_I$ and $\Psi_{100}$, where the relationships between reference and predicted data did not hold. We found significant and strong correlations among reference and predicted field data, with the exception of some correlations related with $\Psi_w$ and $\Psi_{100}$ ($\Psi_w$ with $T_I$ and $\Psi_{100}$; $\Psi_{100}$ with SLA). Overall, we found more and stronger correlations using predicted data than when using reference measurements (Supplemental Tables S3 and S4).

Using the reference values only, few significant correlations found in the greenhouse were confirmed in the field (A with E and $g_s$, E with $g_s$, CI with WUE$_{in}$, WUE with $T_I$, and $\Psi_w$ and $\Psi_{100}$, Supplemental Table S3). Using reference values, greenhouse measurements of A were positively related with the normalized differential vegetation index (NDVI) and a scaled photochemical reflection index (sPRI, $r$ of 0.41 and 0.52, respectively, $P < 0.05$, $n = 155$), but not the normalized differential water index (NDWI, $r$ of 0.24, $P > 0.05$, $n = 155$). Using reference measurements collected in the field, however, A was not related with NDVI, sPRI, or NDWI ($r$ of 0.18, -0.25, and 0.05, $P > 0.05$ $n = 36$). Greenhouse reference values of RWC, Suc and $\Psi_w$ were positively related to NDWI; although statistically significant, the relationships for all three variables with NDWI were weak ($r$ of 0.22, 0.26, and 0.17, respectively, $P < 0.05$, $n = 173$ for RWC and Suc and $n = 169$ for $\Psi_w$).

**Leaf trait responses under different drought conditions**

A three-way ANOVA, including yield stability, water treatment, and stage (Table 3) revealed that Suc and SLA were generally higher (4%) in genotypes with high yield stability. No other traits were significant for yield stability (Supplemental Figure S1). We found significant water treatment effects for all leaf traits except WUE$_{in}$, $T_I$ and TSD (Table 3). In general, A, E, $g_s$, WUE$_{in}$, ChlSPAD, $\Psi_w$ and RWC decreased under mild drought (MD) (-75%, -75%, -79%, -22%, -5%, -45% and -10%, respectively) and even more under severe drought (SD) (-86%, -85%, -88%, -32%, -12%, -64%, -20%, respectively). Overall, $\Psi_w$ and Suc decreased approximately 12% and 6%, respectively, and CI increased $ca$. 65% under MD and SD. $\Psi_{100}$ and SLA increased only under SD (+11% and +9%, respectively, Supplemental Figure S1). We also found significant stage effects for all leaf traits except $\Psi_w$ (Table 3), with A, E, $g_s$, WUE$_{in}$, ChlSPAD, $\Psi_w$, RWC, Suc and SLA generally decreasing (-39%, -30%, -35%, -38%, -25%, -2%, -5%, -12%, -24% and -16%, respectively) and CI, $T_I$, $\Psi_{100}$ and TSD increasing (+85%, +13%, +9% and +14%, respectively) at the later V10 developmental stage (Supplemental Figure S1). The magnitude of response, however, varied across water treatments and was different for different genotypes.

We found no significant yield stability × water treatment or yield stability × stage interactions, whereas we found significant water treatment × stage interactions for A, E, $g_s$, CI, WUE$_{in}$, WUE$_{in}$, $\Psi_w$, $\Psi_{100}$ and RWC (Table 3). Early in development (V6), A, E and $g_s$ were decreased more under SD than under MD, whereas at the later (V10) stage, the effects were similar under MD and SD. A similar behavior was observed for $\Psi_w$, which had the lowest values at the first developmental stage under SD. CI and WUE decreased under water deprivation only at the V10, and the degree of effect of the stress was similar in both MD and SD treatments. WUE$_{in}$ was increased in MD and SD plants at the first developmental stage but decreased in both treatments at the second developmental stage. $\Psi_{100}$ increased in V10 plants under SD. The decrease of RWC was greater under SD than MD only at the V10 stage (Supplemental Figure S1). Only ChlSPAD showed a significant yield stability × water treatment × stage interaction. Genotypes with high yield stability maintained high ChlSPAD values under MD at the V6, but not V10, stage (Table 3, Supplemental Figure S1).

A second three-way ANOVA, including genotype, water treatment, and stage (Table 4), revealed specific genotype characteristics for several traits. For A, E, $g_s$ and Suc, PI601361 (drought-sensitive) and PI543842 (drought-tolerant) had higher values, whereas PI55936 and PI601438 (both drought-sensitive) had lower values across all water treatments. For ChlSPAD and TSD, we found higher values in PI6011361 and PI543842 but lower values in Ames27193 (drought-tolerant) across water treatments. When averaged across water treatments, PI6011361 showed higher $\Psi_w$ values that were statistically significantly different from those of Ames27193, PI601438 and PI55936 (drought-tolerant). Ames27193, PI601438 and PI55936 had...
lower SLA values than PI55935 (Supplemental Figure S1, Supplemental Table S5). We also found significant genotype × water treatment interactions for A, E, ChlSPAD, \( \Psi_w \) and \( \Psi_{e100} \) (Table 4). Under MD, PI6011361 and PI543842 had higher values of A, E and ChlSPAD than the other genotypes, whereas no genotypic differences for these traits were observed under SD. PI543842 and PI601438 had a greater decrease in \( \Psi_w \) under SD, whereas no genotypic differences were found under MD. Although genotypic differences of \( \Psi_{e100} \) were found for controls with higher values in PI559936 and PI6011361 and lower values in PI601438 and Ames27193, no differences among genotypes were found under either MD or SD (Supplemental Figure S1). Significant genotype × stage interactions were found for ChlSPAD, \( \Psi_w \), SLA and TSD (Table 4). PI6011361 showed numerically the highest ChlSPAD values at both stages of analysis, but it was significantly different from only Ames27193 at V6, while it was significantly different from PI55935, PI559936 and PI6011361 at V10. At V9, Ames27193 and PI6011361 and PI6011361 demonstrated higher \( \Psi_w \) values than PI601438, whereas no genotypic differences in \( \Psi_w \) were observed at V10. SLA was higher in PI559935 than PI559936, PI601438 and Ames27193, whereas no genotypic differences were observed at V10. No genotypic differences were observed at V6 in terms of TSD, while at V10 only PI601438 and Ames 27193 remained at the levels reported at V6 as all the other genotypes increased (Supplemental Figure S1). A significant genotype × water treatment × stage interaction was found only for WUEin and ChlSPAD (Table 4, Supplemental Figure S1). PI6011361 (drought-sensitive) and PI559936 (drought-sensitive) were the only genotypes where WUEin remained high at V10, and specifically so under MD and SD. PI6011361 (drought-sensitive) showed an ability to maintain optimal ChlSPAD values at V6 under water stress, whereas PI543842 (drought-tolerant) showed this response at V10. The greatest reductions in ChlSPAD under MD were observed in Ames27193 (drought-tolerant) at V6 and in PI601438 (drought-sensitive) at V10.

**DISCUSSION**

We describe a non-destructive approach by which plant responses to water availability can be monitored using reflectance spectroscopy. By combining reflectance measurements, standard physiological and anatomical measurements and statistical modelling, we demonstrate the potential of using spectral data to estimate maize leaf traits that often change under drought stress. This documents the potential of spectroscopy to estimate a large number of drought-related leaf features in maize and also highlights the possibility to estimate water potential traits in C4 plants.

Photosynthesis is a key plant process that is affected by drought, primarily through stomatal and mesophyll limitations (Pinheiro and Chaves, 2010). Standard measurements of light- and CO2-saturated photosynthesis, however, arelogically challenging, potentially taking 20-30 minutes per leaf as the leaf has to acclimate to the cuvette conditions. Hyperspectral approaches present a rapid alternative to standard reference measurements and have been used previously to estimate photosynthetic activity in plants both indirectly, via xanthophyll cycling (PRI; Gamon et al., 1997; Peñuelas et al., 2011) and directly, by predicting specific traits from spectral data, mainly RuBP, \( V_{cmax} \) and \( J_{max} \) (e.g., Serbin et al., 2012; Ainsworth et al., 2014, Heckmann et al., 2017; Yendrek et al., 2017).

The spectral region best predicting gas-exchange traits was 500–900 nm. This wavelength region contains wavelengths with pigment absorption features (Merzlyak et al., 2003) as well as the red-edge (700–750 nm; Mutanga and Skidmore, 2007), a spectral region strongly influenced by chlorophyll content (Filella and Peñuelas, 1994; Clevers et al., 2004; Smith et al., 2004; Zarco-Tejada et al., 2004) and plant stress condition (Smith et al., 2004; Mutanga and Skidmore, 2007; Cotrozzi et al., 2017b). The importance of spectral features in this wavelength range for assessing photosynthetic processes has been reported for several plant species (e.g., Gamon et al., 1997; Merzlyak et al., 2003; Serbin et al., 2012; Yendrek et al., 2017). The similarities in wavelength ranges of importance across the different models we report highlight that changes in pigments are important for spectral detection of plant stress (Gamon et al., 1997; Merzlyak et al., 2003). Here we also show that the range of traits estimable (e.g., gas-exchange) can be expanded using spectroscopy to better understand specific physiological responses to water stress.
We found that the most important spectral region for prediction of SPAD-based chlorophyll estimates was 600–900 nm. Wavelengths between 700 and 750 showed the highest VIP values reported for predicting SPAD-based chlorophyll, highlighting the stronger relationship between SPAD values and the red-edge, compared with other traits.

A, E, and gs were well predicted, followed by WUEi and ChlSPAD, and then by Ci and WUEin. These outcomes suggest that physiologically processes derived from calculations of other measurements (i.e., predictions were used in calculation of processes) lost prediction accuracy, compared with processes that were directly predicted (e.g., A, E, gs). The performance of the model predicting A was confirmed by the strong and positive relation with NDVI and sPRI, two spectral indices related to photosynthetic activity (Gamon et al., 1995, 1997). Using predicted values to calculate WUEin (WUEincalc), compared with predicting WUEin directly, as a test case (WUEincalc vs WUEin), the prediction accuracy further decreased when using predicted values to calculate WUEincalc (i.e., predicted A and gs) opposed to directly predicting WUEincalc using spectral data alone. This outcome is likely due to the propogation of error from the individual predictions in the calculations.

Interestingly, we found excellent prediction performance for Tl ($R^2$: 0.89, for cross-validation). The capability of reflectance spectroscopy to detect Tl changes has been previously reported across a number of C3 species exposed to variable air temperatures (e.g., Serbin 2012). The wavelength range utilized by Serbin (2012) included a much larger range (400-2400 nm), contrasted with the narrower range (500-900 nm) we utilized. The differences between the two outcomes is likely a consequence of the different treatments utilized in the two studies: temperature opposed to water stress. The ability of photosynthetic and accessory pigments to safely dissipate excess light energy through the xanthophyll cycle (Demmig-Adams and Adams, 1996) likely contributes to the ability of spectral data to accurately predict temperature in the current study.

A further explanation in the discrepancy between wavelength regions from the current study and those from Serbin (2012) is likely due to the different photosynthetic pathways, foliar anatomical structure, and experimental treatments used. Moreover, the SWIR region is strongly sensitive to water content, given the prominent water absorption features in this spectral region (Curan 1989), and the inclusion of different watering treatments in the current study may have altered the influence of this spectral region on predicting leaf temperature, shifting focus on excess energy dissipation via shifts in pigment profiles.

Measurements of water status can be broadly divided into either the amount of leaf water or leaf energy status (Jones, 2007). Reflectance spectroscopy likely utilizes information from both approaches because vegetation reflectance is influenced by the amount of water as well as by the composition and concentration of osmolytes that affect variation in $\Psi_w$, and ultimately in $\Psi_w$ (Cortozzi et al., 2017a). We found that three spectral regions best predicted the investigated water-related traits. Unexpectedly, $\Psi_w$, which is currently the most widely used parameter to estimate the water status of plants exposed to drought (González-Fernández et al., 2015), was best predicted using the full spectral range (400–2400 nm). This is in contrast with previous findings (Santos and Kaye, 2009; De Bei et al., 2011; Cotrozzi et al., 2017a) that the NIR-SWIR region best predicts $\Psi_w$. However, $\Psi_w$ is the sum of a number of components, including dissolved solutes ($\Psi_a$), the pressure potential ($\Psi_p$, equal to the hydrostatic pressure) and the gravitational potential ($\Psi_g$), ignorable except in tall trees, and is also influenced by photosynthetic regulation (Jones, 2007). Thus, $\Psi_w$ is the outcome of the coordination of a number of underlying factors, each of which can potentially interact with spectral regions differently. We feel the inconsistency among other studies and ours in determining the best spectral region for predicting $\Psi_w$ is likely due to the anatomical differences between C3 and C4 species, photosynthetic pathways, and severity and range of stress investigated.

For other modeled water status-related traits, the best predicting models were derived from the spectral region dominated by water content and outside of wavelengths commonly associated with pigments. As we expected, models of RWC and Suc, which are the measurements based on the amount of leaf water, performed better by using the whole NIR-SWIR spectral region. Agreeing with Cotrozzi et al. (2017a),
standardized coefficients and VIP values effectively highlighted two important water absorption features of the leaf reflectance profile (1350–1500 and 1900–2100 nm). The ability of vegetation spectroscopy to estimate RWC in maize has been previously reported, but primarily through the use of spectral indices (Schlemmer et al., 2005; Zygielbaum et al., 2009; Zhang and Zhou, 2015). While we also found statistically significant relationships between RWC, Suc and \( \Psi_w \) with NDWI, a spectral index widely used to estimate plant water content (Gao, 1996), the relationships were weak and had substantially less explanatory power than the PLSR predicted estimates we generated, likely due to the limited inclusion of spectral data with strong water absorption features. We found that the wavelength region that best predicted \( \Psi_s \) and \( \Psi_{s100} \) was primarily the SWIR region and excluded the minor water absorption features centered at 970 nm and 1200 nm. By eliminating the NIR portion of the spectrum, we potentially amplified the contribution of osmolytes to the prediction of \( \Psi_s \) and \( \Psi_{s100} \) in maize leaves. Multiple studies (Shetty and Gislum, 2011; Rubert-Nason et al., 2013; Asner and Martin, 2015; Ramirez et al., 2015; Cotrozzi et al., 2017a) have reported that wavelength regions important for predicting non-structural carbohydrates and other foliar osmolytes using spectroscopy align or closely overlap with the more important wavelengths we found for optimal model prediction of \( \Psi_s \) and \( \Psi_{s100} \). This finding suggests that when SWIR data are available, they can provide a more accurate representation of foliar water content than indices alone. Scaling this approach to airborne data, however, may be challenging because of the noise generated by atmospheric water in the spectral region of strong foliar water absorption.

In the current study, estimation of \( \Psi_w \) was weak for severely drought-stressed plants (\( \Psi_w < -2.0 \) MPa), converse to similar data in live oak reported by Cotrozzi et al. (2017a). Given the sub-optimal model performance, we encourage caution when interpreting results from a narrow range of values of \( \Psi_w \), \( \Psi_s \) and \( \Psi_{s100} \). Regardless of this limitation, and considering that measurements of these traits with standard methods (pressure chamber and vapor pressure osmometer) have several constraints in that they are destructive, user-dependent and point based (Santos and Kaye, 2009; González-Fernández et al., 2015), we propose spectroscopy as a rapid and non-destructive approach to unbiasedly estimate water, increasing data collection efforts over larger spatial and temporal scales.

Leaf anatomical traits (e.g., leaf thickness and stomatal density) play a pivotal role in plant tolerance to drought (Zhao et al., 2015; Wellstein et al., 2017), yet measurements of these traits are time-consuming and destructive. By using the SWIR region, we observed a strong predictive ability for SLA (\( R^2 \): 0.83 for cross-validation). This outcome was comparable with outcomes reported by Yendrek et al. (2017, \( R^2 \): 0.68–0.78) using spectral data collected from diverse inbred and hybrid lines grown at ambient and elevated ozone concentrations in the field. Standardized coefficients and VIP metrics for SLA highlighted the importance of the wavelengths related to the water content of leaves. As we expected, the weakest predictive ability among the models presented in this study was for stomatal density. For the nature of the trait, we found it difficult to estimate stomatal density from leaf reflectance, as suggested by the variability in the model coefficient and VIP weighting. Stomata are localized only on the epidermal surfaces of leaves, and while the interaction of light with the epidermis contributes to overall reflection profiles, reflectance measurements are also affected by the leaf tissue as a whole, including the palisade and cuticular layers, and this relationship likely impeded stomatal density prediction from spectral data.

An emergent outcome of this study is the advancement of conceptual approaches of chemometric modelling by examining the influence of multiple modelling approaches and different reference measurement collections on model outcomes. Comparing different modelling approaches, we found that performance of PLSR was always higher or comparable with the performance of the Lasso approach. The reason for the higher performance of PLSR than Lasso likely has to do with the trait response being modelled. Responses that contain singular relationships with spectra (e.g., predictors can converge to a few highly important wavelengths, such as pigments) can be modelled after removing a large portion of the spectrum, as done by Lasso through penalizing intercorrelated variables. We also found that Lasso lost prediction accuracy when
smaller spectral ranges are used, opposed to starting with full spectrum data. Physiological processes and
other phytochemical compounds may need larger portions of the spectrum for successful modelling because
the necessary absorption features are contained within numerous and different spectral regions associated
with the components parts of these processes. The contributions of smaller coefficients, which are included
in PLSR but removed in Lasso, likely improve prediction accuracies in PLSR because they provide related
information to the coefficient of absorption features that individually are sub-optimal, but cumulatively
contribute more than individual wavelengths alone.

Our measurements of spectral and reference measurements from both controlled and field environments
revealed three outcomes. First, greenhouse-based models allowed us to capture a greater proportion of trait
variation than using only field-grown plants because plants were exposed to a greater and wider range of
stress conditions. Second, the prediction accuracy of models built using both greenhouse and very few field
measurements was similar with predictions made using spectra on either greenhouse or field samples, as long
as the trait values in field samples were within the prediction range of models built using greenhouse
collections. Third, the prediction of field responses was dramatically compromised if using predictive
models created using spectral data collected from only greenhouse collections. These outcomes demonstrate
that field collections are a necessary compliment to controlled environment studies if the intent is to use
models for phenotyping field plant stress responses.

We found that few of the physiological and anatomical traits measured in this study were correlated with
yield stability for the specific maize hybrids used in the present study. Only the regulation of chlorophyll
content, determined as SPAD, varied between genotypes with high or low yield stability conditions. Indeed,
we commonly found that a genotype within each drought-sensitivity class (i.e., tolerant or sensitive)
exhibited inconsistent physiological responses to water stress. Both drought-sensitive and drought-tolerant
genotypes exhibited higher photosynthesis, transpiration rates and ChlSPAD under moderate drought stress,
and some drought sensitive genotypes had the lowest Ψw values under water stress. We found a significant
three-way interaction (yield stability class by water stress by ascession) for only ChlSPAD and intrinsic water
use efficiency with two drought-sensitive genotypes able to maintain this status at optimal levels under water
stress at later developmental stages. The lack of a relationship between the traits contributing to a statistically
significant response and yield stability is likely due to the speed of the onset of stress and differences in
water availability in the containers, compared to field conditions where yield stability was assessed.

However, we found that the physiological and anatomical responses measured in the maize hybrids in
response to water stress were well predicted using hyperspectral data, as almost all the statistical outputs for
the correlations obtained with reference measurements were confirmed in both greenhouse and field samples.
This is in agreement with those of others for spectral predictions of maize traits under differential
environmental treatments (Yuan et al., 2016, Heckmann et al., 2017; Yendrek et al., 2017).

CONCLUSIONS

Here we demonstrate that reflectance spectroscopy provides a rapid, non-destructive approach to accurately
quantify physiological and anatomical functional traits associated with water relations in maize using a
single spectral measurement. Importantly, this suggests that the predictions from spectral data can be used in
place of standard reference collections. Moreover, this approach can dramatically increase data collection
from a larger number of individual genotypes and plants, in both controlled and field conditions, than
reference measurements alone. The increase in data volume collected using hyperspectral data, coupled with
similarity in the outcomes of statistical analyses used to measure responses across treatments, should
promote the implementation of more complex experimental designs that can provide greater insight into the
genetic, environmental, and gene-by-environment interactions that enhance and repress agricultural
production. While logistical challenges (e.g., solar angle or atmospheric interference) exist, leaf-level
spectroscopy can be effectively used as ground reference or training input for airborne-based platforms and scaled to field and landscape levels (Serbin et al., 2015; Meacham-Hensold et al., 2019). Our ability to detect crop stress responses may play a pivotal role in precision agriculture and advancing trait-based plant breeding.

**MATERIALS AND METHODS**

**Plant material and experimental design**

**Greenhouse experiment**

Experiments were conducted in the Purdue University Horticulture Plant Growth Facility (40°25’15’’N, 86°54’51’’W, 611 m a.s.l.), West Lafayette, IN, USA. The USA is the world leader in maize (*Zea mays*) production, and the US Midwest represents the major maize production region of North America (Grace et al., 2011; Araus et al., 2012). Seeds of six maize hybrids with different yield stability responses under drought (drought-tolerant: Ames27193xPHP02, PI543842xPHP02 and PI559935xPHP02; drought-sensitive: PI559936xPHP02, PI6011361xPHP02, PI601438xPHP02; M. V. Mickelbart and M. R. Tuinstra, unpublished data, Supplemental Table S5) were planted in a growing media containing a mixture of Fafard 52® Mix Metro-Mix 852® RSi professional growing mix (Sun Gro Horticulture Inc., Agawam, MA, USA) and Turface Athletics MVP® conditioner (PROFILE Products LLC, Buffalo Grove, IL, USA) (2:1 in volume) in 7.5-L black plastic containers. After planting, containers were regularly irrigated to field capacity with a fertilizer mix (400 ppm N) using 21-5-20 Peters Excel fertilizer (ICL Specialty Fertilizers, Dublin, OH, USA). The greenhouse day and night mean temperatures were 26 and 20 °C, respectively; and maximum day and night RH were ~60 and ~50%, respectively.

Following a randomized block design, plants were then assigned to two different sets each composed of 15 seedlings per genotype, and sub-assigned to three different water treatments: irrigated every day to field capacity (well-watered, WW), water withheld for four days (mild drought, MD) before measurements (dbm), or water withheld for nine dbm (severe drought, SD). The water treatments were applied at two developmental stages. Both sets of plants experienced the first episode of different watering regimes, but only the V6-stage leaf of the first set of plants was measured for Stage 1. Following the second round of different water treatments, the V10 leaf of the second set of plants was measured for Stage 2. On the sixth or tenth leaf (i.e. the youngest fully-expanded leaves at the two stages of analysis) of each plant, we collected measurements in the following order: gas-exchange, leaf greenness, reflectance and water potential, then leaf portions were collected for the determination of other water status traits and thickness, as well as imprinted slides for stomatal density (for a total of 180 leaf samples; Supplemental Figure S2). These specific traits were selected because they are commonly investigated to evaluate the effects of drought on plants. All measurements were performed between 10.00 and 12.00 h.

**Field experiment**

Field activities were conducted at the Agronomy Center for Research and Education of Purdue University (ACRE, 40°28’22”N, 86°59’37”W, 216 m a.s.l.), West Lafayette, IN, USA. The soil type is a Chalmers silty clay loam. Average soil pH, organic matter, exchangeable P and available K were 5.4, 2.5%, 39 lbs/acre and 170 lbs/acre, respectively. The previous crop was soybeans. Tillage consisted of deep ripping the previous fall and field cultivation in the spring prior to planting. The same six hybrids used for the greenhouse experiment were planted in a thirty-six randomized block design (12×3, six replications per hybrid). Each block was 5.334 m long and 3.048 m wide with 4 rows and 0.762 m row spacing. Each row of each block contained ca. 25 plants. Planting date was June 1, 2017. All grass and broadleaf weeds in the plot areas were controlled with a pre-emergent residual herbicide Bicep II Magnum (S-metolachlor and atrazine). All maize seeds were treated in a similar manner with AcceleronTM (Difenoconazole, Fludioxonil,
Mefenoxam, and Thiamethoxam). Force 3G (Tefluthrin) was soil-applied at planting to control corn rootworm (*Diabrotica virgifera virgifera*).

On 19 July 2017, measurements of gas exchange, leaf greenness, reflectance, water and osmotic potentials, and thickness were collected on the 9th leaf (the youngest fully-expanded leaf) of the plant located in the center (12th plant of the second row) of each of 36 blocks (36 leaf samples, six leaves per hybrid) from 11.00 to 16.00 h, following the same procedure adopted for the greenhouse experiment. Weather data were collected from Purdue University Indiana State Climate Office at station ‘ACRE-West Lafayette’ (http://www.iclimate.org/). Total precipitation from June 1, 2017 to 19 July 2017 was 257.53 mm, and averaged daily, maximum, and minimum temperatures were 22.7, 27.6, and 16.3 °C, respectively. No supplemental irrigation was applied.

The collection of data in both greenhouse and field environments was performed to achieve the second main goal of the present study (i.e., compare the performance of models built using only greenhouse samples with models including also field samples) as schematized in Supplemental Figure S3. Further details are reported below.

**Gas exchange and chlorophyll content**

Net CO₂ assimilation (A), transpiration (E), stomatal conductance (gₛ), leaf intercellular CO₂ concentration (Cᵢ) and leaf temperature (Tᴸ) were determined using a LI-6400XT portable photosynthesis system equipped with a 6400-02B LED light source (Li-Cor, Inc., Lincoln, NE, USA), operating at 400 ppm CO₂ concentration and saturating light conditions (1700 µmol m⁻² s⁻¹ PAR). Instantaneous (WUEᵢ) and intrinsic (WUEᵢₙ) water-use efficiency were calculated as A/E and A/gₛ respectively. A SPAD 502 meter (Minolta, Osaka, Japan) was used to determine leaf greenness (ChlSPAD). Three measurements per leaf were made, and the mean of these measurements was recorded.

**Collection of leaf spectra**

Full range (350–2500 nm) reflectance profiles of maize leaves were collected using a SVC-1024i spectroradiometer (Spectral Vista Corporation, Poughkeepsie, NY, USA) using a leaf-clip with an internal halogen light source attached to a plant probe. Integration time (i.e., the length of time that the detectors are allowed to collect photons before passing the accumulated charge to the A/D converter for processing) was set at two seconds. Measurements were made on three and five areas of the leaf adaxial surface for each greenhouse and field leaf, respectively, with one measurement per area, and all measurements were combined to produce an average leaf spectrum. The relative reflectance of each leaf was determined from the measurement of leaf radiance divided by the radiance of a white reference panel internal to the leaf clip, measured every 12 spectral collections.

**Water status and leaf thickness**

Ψᵥ was measured on the distal portion of the leaf (15–20 cm) that was cut with a sharp razor blade, inserted into a rubber stopper and then placed in a Scholander-type pressure chamber (Model 600; PMS Instrument Company, Corvallis, OR, USA), following the precautions suggested by Tiekstra et al. (2000). To determine Ψᵣ, a portion of the leaf used for Ψᵥ was placed in a mesh insert in a microcentrifuge tube, immersed in liquid nitrogen and stored at -20 °C until processing. Solute concentration was determined with a vapor pressure osmometer (Wescor 5500; Wescor Inc., Logan, UT, USA) as reported in Stanton and Mickelbart (2014). RWC and Suc were determined on another portion of the same leaf. Following a standardized procedure (Stanton and Mickelbart, 2014), RWC was calculated as FW-DW/TW-DW, where FW, DW and TW are fresh, dry and turgid weights, respectively. Leaf Suc was calculated as FW/DW. The Ψᵣ₁₀₀ was calculated as Ψᵣ × RWC.
After determination of TW, the leaf portion used for determining RWC was scanned prior to drying, and its leaf area (LA) was determined using the ImageJ×1.38 software (National Institutes of Health, Bethesda, MD, USA). Specific leaf area (SLA) was calculated as LA/DW.

Stomatal density

A leaf surface imprint method was used to determine stomatal density (SD; Weng et al., 2012). Abaxial (Ab) and adaxial (Ad) epidermal cell outlines of leaves were imprinted onto cyanoacrylate droplets that were placed onto glass slides. Images were taken under 10× magnification using a Nikon-OptiPhot2 microscope. Stomata were counted in an area of 0.474 mm². For each slide, four independent leaf areas were counted and averaged for each biological replicate. TSD was calculated as Ab SD+Ad SD.

Model calibration and validations

Using both greenhouse and field samples, we generated models to predict leaf traits from untransformed reflectance profiles using PLSR (Wold et al., 2001). When predictor variables are highly correlated, as is the case with hyperspectral data, classical regression techniques can produce unreliable coefficients and error estimates (Grossman et al., 1996). In contrast to standard regression techniques, PLSR reduces a large number of collinear predictor variables into relatively few, uncorrelated latent variables, and has become the preferred method for chemometric approaches (Bolster et al., 1996; Atzbeger et al., 2010; Couture et al., 2016, 2013; Cotrozzi et al., 2017b). To avoid potential overfitting the PLSR-model, the number of latent variables used was based on reduction of the predicted residual sum of squares (PRESS) statistic (Chen et al., 2004) using leave-one-out cross-validation. Once minimized, the final set of extracted components was combined into a linear model predicting leaf traits based on leaf spectral profiles.

Model performance was evaluated by conducting 500 randomized permutations of the data sets using 80% of the data for calibration (i.e., training) and the remaining 20% for cross-validation (i.e., testing) with random resampling for each permutation. For each permutation, we tracked the $R^2$, the overall error rate (RMSE), the percentage of error relative to the data range (NRMSE) and bias to assess model performance when applied to the validation data set. These randomized analyses generated a distribution of fit statistics allowing for the assessment of model stability as well as uncertainty in model predictions. We further determined the strength contribution of PLSR loadings by individual wavelengths using the VIP selection statistic. The VIP statistic evaluates the importance of individual wavelengths in explaining the variation in both the response and predictor variables, where larger weightings confer greater value to contribution of individual wavelengths to the predictive model (Wold et al., 2001; Chong and Jun, 2005). Using A, Ψw, Ψπ and SLA as testing traits, we also compared the performance of models built with both greenhouse and field samples and models including only greenhouse data (Supplemental Figure S3b).

We additionally performed external validation by applying PLSR-coefficients on a dataset distinct from the one used for calibration and cross-validation, including again both greenhouse and field samples (ca. 40% of the full dataset). Five-hundred randomized permutations were conducted, and relations between predicted and observed values were tested by regression analysis. Fit statistics ($R^2$, RMSE, bias, NRMSE) were again used to assess model estimation accuracy. Using A, Ψw, Ψπ and SLA as testing traits, we also compared the estimation efficacy for these traits in field samples between the models built with both greenhouse and field samples and models including only greenhouse data (Supplemental Figure S3b).

Before building the final models, we developed preliminary models to identify wavelength regions associated with the trait of interest and to identify poorly predicted outliers on either the reference or target measurements. Final wavelength regions included individual wavelengths at 1 nm intervals, thus maintaining high-density spectral information in regions associated with traits, but excluding wavelengths that contribute error to the predictions likely because of lack of absorption features. Prediction residuals were used to identify potential outliers. Spectral data of outliers were further examined for errors, detectable from elevated
reflectance in the VIS wavelengths or spectral jumps in the NIR region that occur when the leaf clip is not fully closed. Reference data of outliers were also examined for extremes in the data distribution (Couture et al., 2016, 2013; Cotrozzi et al., 2017b; Marchica et al., 2019). The same approach was followed to remove outliers from the data set used for external validations. Outliers removed accounted for 12-13% of the initial data.

To test another multivariate statistical approach, we also analyzed the same data sets using least absolute shrinkage and selection operator (Lasso, Tibishirani, 1996, 2011). The aim of the Lasso approach is to improve prediction accuracies by minimizing, or shrinking, coefficients to to be less than a fixed, upper-bounded value and removes coefficients of intercorrelated variables by setting them to zero, providing variable selection. The same as our PLSR approach, Lasso model performance was evaluated by conducting 500 randomized permutations of the data sets using 80% of the data for calibration and the remaining 20% for cross-validation; and for each permutation, we tracked $R^2$, RMSE, NRMSE and bias statistics. The modelling approach and data analyses were performed using the ‘pls’ (Mevik et al., 2016) and ‘glment’ (Friedman et al., 2010) packages in R (www.r-project.org) for PLSR and Lasso, respectively.

Spectral indices

Some widely used spectral indices thought to be related with plant health and water status were also calculated: photochemical reflectance index (PRI), an indicator of photosynthetic radiation use efficiency, \((R_{531}-R_{570})/(R_{531}+R_{570}),\) Gamon et al., 1997). To avoid negative values of PRI, values were scaled as sPRI=(PRI+1)/2 as reported by Letts et al. (2008); normalized differential vegetation index (NDVI), an indicator of leaf greenness, photosynthetic activity and plant health \((R_{780}-R_{570})/(R_{780}+R_{570}),\) Gamon et al., 1995); and normalized differential water index (NDWI), an indicator of vegetation liquid water content, \((R_{857}-R_{1241})/(R_{857}+R_{1241})\) (Gao, 1996). Rx indicates reflectance at x nm wavelength.

Statistical analyses

We first determined the effects of yield stability under drought, water treatment, stage and their interactions on leaf traits of greenhouse samples, by using a three-way analysis of variance (ANOVA) following the model

\[ Y_{ijk} = \mu + Y_s i + W_j + S_k + Y_s W_{ij} + Y_s S_{ik} + W_j S_{jk} + Y_s W_j S_{ijk} + e_{ijk}. \]

Then, we similarly determined the effects of genotype, water treatment, stage and their interaction on leaf traits of greenhouse samples, using a three-way ANOVA following the model

\[ Y_{ijk} = \mu + G_i + W_j + S_k + G W_{ij} + G S_{ik} + W_j S_{jk} + G W_j S_{ijk} + e_{ijk}. \]

In these models, \(\mu\) represents the mean, \(Y_s\) is yield stability under drought level \(i\), \(G\) is genotype level \(i\), \(W\) is water treatment level \(j\), \(S\) is stage level \(k\), and \(e_{ijk}\) represents the error term.

Relationships among predicted leaf traits from greenhouse, field or greenhouse and field samples were evaluated using Pearson’s correlations. Pearson’s correlations were also used to test the relations among A with NDVI, sPRI and NDWI, as well as \(\Psi_w\), RWC and Suc with NDWI. For relationships among A and spectral indexes, data were separated into either greenhouse or field data; for relationships among \(\Psi_w\), RWC, Suc, and NDWI only greenhouse data were available. Statistical analyses (included regression analyses for external validations of models) were performed in JMP 13.2 (SAS Institute Inc., Cary, NC, USA). The normality of data was preliminary tested by the Shapiro-Wilk W test. Effects with \(P<0.05\) were considered statistically significant.

Supplemental Data

Supplemental Figure S1. Observed leaf traits of six hybrids of maize characterized by different yield stability under drought exposed to different water treatments at different developmental stages.
Supplemental Figure S2. Experimental scheme of the greenhouse experiment.

Supplemental Figure S3. Modellong approach schematic.

Supplemental Table S1. Metadata and performance of the preliminary PLSR-models used for the estimation of leaf traits by spectral data.

Supplemental Table S2. Metadata and performance of the Lasso-derived models used for the estimation of leaf traits by spectral data.

Supplemental Table S3. Pearson’s correlation matrix describing relationships among leaf traits of greenhouse and field samples by observed standard measurements.

Supplemental Table S4. Pearson’s correlation matrix describing relationships among leaf traits of greenhouse and field samples predicted by PLSR-models.

Supplemental Table 5. Identification and description of hybrids used in the current study.

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### TABLES

#### Table 1. Model performance statistics for calibration (C) and cross-validation (CV) data generated via cross-validation using 500 random permutations of the data with 80% used for C and 20% used for CV for models predicting leaf traits from maize spectra. Fit ($R^2$); root-mean-square error (RMSE); normalized root-mean-square error (NRMSE). Data are shown as mean ± standard deviation (5th–95th percentile). Trait abbreviations: A, net CO$_2$ assimilation ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$); E, transpiration (mmol H$_2$O m$^{-2}$ s$^{-1}$) g$_s$, stomatal conductance (mol H$_2$O m$^{-2}$ s$^{-1}$); Ci, intercellular CO$_2$ concentration (µmol mol$^{-1}$); WUE$_{in}$, instantaneous water use efficiency (µmol CO$_2$ mmol$^{-1}$ H$_2$O); WUE$_{in}$, intrinsic water use efficiency (µmol CO$_2$ mol$^{-1}$ H$_2$O); $T_b$, temperature of adaxial leaf surface (°C); ChlSPAD, chlorophyll content; $\Psi_w$, leaf water potential (-MPa); $\Psi_s$, leaf osmotic potential (-MPa); $\Psi_{\delta 100}$, leaf osmotic potential at full turgor (-MPa); RWC, relative water content (%); Suc, succulence (g g$^{-1}$); SLA, specific leaf area (cm$^2$ g$^{-1}$); TSD, total stomatal density (mm$^2$).

| Trait | C  | R$^2$ | RMSE | Bias | NRMSE |
|-------|----|------|------|------|--------|
|       | C  | CV   | C    | CV   | C      | CV   |
|       |    |      |      |      |        |      |
| A     | 0.92±0.01 | 0.84±0.05 | 4.61±0.23 | 6.61±0.93 | 0.00±0.0 | 0.28±1.60 | 9 13 |
|       | (0.91–0.94) | (0.74–0.91) | (4.24–4.95) | (5.09–8.15) | (0.00–0.0) | (-2.43–2.95) |
| E     | 0.92±0.01 | 0.83±0.05 | 0.77±0.04 | 1.15±0.15 | 0.00±0.0 | 0.02±0.27 | 9 13 |
|       | (0.9–0.93) | (0.74–0.90) | (0.71–0.82) | (0.91–1.40) | (0.00–0.0) | (-0.41–0.48) |
| g$_s$ | 0.86±0.01 | 0.73±0.08 | 0.05±0.00 | 0.08±0.01 | 0.00±0.0 | 0.00±0.0 | 9 15 |
|       | (0.84–0.89) | (0.59–0.83) | (0.05–0.06) | (0.06–0.10) | (0.00–0.0) | (-0.03–0.03) |
| C$_i$ | 0.69±0.03 | 0.51±0.17 | 40.43±1.88 | 50.06±7.52 | 0.00±0.0 | -0.36±11.76 | 13 16 |
|       | (0.63–0.74) | (0.22–0.74) | (37.41–43.37) | (37.50–63.03) | (0.00–0.0) | (-19.25–18.11) |
| WUE$_{in}$ | 0.85±0.03 | 0.69±0.17 | 0.53±0.02 | 0.69±0.10 | 0.00±0.0 | 0.02±0.16 | 7 9 |
|       | (0.80–0.88) | (0.36–0.88) | (0.50–0.56) | (0.55–0.86) | (0.00–0.0) | (-0.26–0.29) |
| T$_b$ | 0.64±0.04 | 0.44±0.17 | 21.35±0.92 | 26.57±4.01 | 0.00±0.0 | 0.10±6.11 | 12 14 |
|       | (0.57–0.70) | (0.17–0.71) | (19.66–22.62) | (20.41–33.38) | (0.00–0.0) | (-10.59–9.06) |
| ChlSPAD | 0.95±0.00 | 0.89±0.03 | 0.52±0.02 | 0.79±0.11 | 0.00±0.0 | 0.01±0.18 | 5 10 |
|       | (0.94–0.96) | (0.82–0.93) | (0.48–0.55) | (0.61–0.96) | (0.00–0.0) | (-0.31–0.27) |
| $\Psi_w$ | 0.75±0.02 | 0.61±0.09 | 1.72±0.06 | 2.22±0.27 | 0.00±0.0 | 0.02±0.53 | 10 13 |
|       | (0.73–0.78) | (0.45–0.75) | (1.61–1.81) | (1.74–2.63) | (0.00–0.0) | (-0.91–0.80) |
| $\Psi_n$ | 0.85±0.01 | 0.63±0.10 | 0.10±0.00 | 0.17±0.02 | 0.00±0.0 | 0.01±0.04 | 9 15 |
|       | (0.83–0.87) | (0.44–0.77) | (0.10–0.11) | (0.13–0.20) | (0.00–0.0) | (-0.06–0.07) |
| $\Psi_{\delta 100}$ | 0.79±0.02 | 0.60±0.11 | 0.06±0.00 | 0.08±0.01 | 0.00±0.0 | 0.00±0.02 | 13 17 |
|       | (0.77–0.82) | (0.40–0.76) | (0.05–0.06) | (0.07–0.10) | (0.00–0.0) | (-0.04–0.03) |
| RWC | 0.77±0.02 | 0.53±0.14 | 0.05±0.00 | 0.07±0.01 | 0.00±0.0 | 0.00±0.02 | 10 14 |
|       | (0.74–0.81) | (0.24–0.73) | (0.05–0.05) | (0.06–0.09) | (0.00–0.0) | (-0.03–0.03) |
| Suc | 0.93±0.01 | 0.90±0.04 | 3.22±0.12 | 3.83±0.48 | 0.00±0.0 | 0.01±0.93 | 7 9 |
|       | (0.92–0.94) | (0.80–0.94) | (3.04–3.43) | (2.92–4.62) | (0.00–0.0) | (-1.51–1.56) |
| SLA | 0.97±0.01 | 0.86±0.05 | 0.15±0.01 | 0.32±0.05 | 0.00±0.0 | -0.01±0.09 | 5 10 |
|       | (0.96–0.97) | (0.77–0.92) | (0.13–0.17) | (0.25–0.40) | (0.00–0.0) | (-0.16–0.11) |
| TSD | 0.99±0.00 | 0.45±0.16 | 1.42±0.19 | 14.28±2.16 | 0.00±0.0 | 0.27±3.30 | 1 12 |
|       | (0.99–1.00) | (0.16–0.68) | (1.10–1.71) | (10.72–17.91) | (0.00–0.0) | (-4.79–5.92) |
Table 2. Model performance statistics for external validation. Averaged model goodness-fit ($R^2$), root-mean-square error (RMSE), bias and normalized root-mean-square error NRMSE (%) for external validation data generated via regression analysis using 500 random permutations of the data for models predicting leaf traits from maize spectra. Trait abbreviations: A, net CO$_2$ assimilation (µmol CO$_2$ m$^{-2}$ s$^{-1}$); E, transpiration (mmol H$_2$O m$^{-2}$ s$^{-1}$) $g_s$, stomatal conductance (mol H$_2$O m$^{-2}$ s$^{-1}$); Ci, intercellular CO$_2$ concentration (µmol mol$^{-1}$); WUE$_i$, instantaneous water use efficiency (µmol CO$_2$ mmol$^{-1}$ H$_2$O); WUE$_{in}$, intrinsic water use efficiency (µmol CO$_2$ mol$^{-1}$ H$_2$O); $T_l$, temperature of adaxial leaf surface (°C); ChlSPAD, chlorophyll content; $\Psi_w$, leaf water potential (-MPa); $\Psi_s$, leaf osmotic potential (-MPa); $\Psi_{s100}$, leaf osmotic potential at full turgor (-MPa); RWC, relative water content (%); Suc, succulence (g g$^{-1}$); SLA, specific leaf area (cm$^2$ g$^{-1}$); TSD, total stomatal density (mm$^{-2}$).}

| Trait | $R^2$ | RMSE | Bias | NRMSE |
|-------|-------|------|------|-------|
| A     | 0.86  | 6.96 | 0.70 | 13    |
| E     | 0.89  | 1.39 | 0.33 | 13    |
| $g_s$ | 0.79  | 0.08 | 0.02 | 15    |
| $C_i$ | 0.52  | 49.54| -11.32| 15    |
| WUE$_i$ | 0.76 | 0.77 | -0.13| 11    |
| WUE$_{in}$ | 0.55 | 24.54| 0.97 | 15    |
| $T_l$ | 0.79  | 1.06 | 0.15 | 18    |
| ChlSPAD | 0.44 | 2.99 | -0.15| 17    |
| $\Psi_w$ | 0.40 | 0.25 | 0.00 | 20    |
| $\Psi_s$ | 0.34 | 0.10 | -0.02| 21    |
| $\Psi_{s100}$ | 0.40 | 0.07 | 0.00 | 16    |
| RWC   | 0.65  | 6.28 | 0.80 | 15    |
| Suc   | 0.74  | 0.36 | -0.04| 14    |
| SLA   | 0.77  | 19.87| -0.88| 12    |
| TSD   | 0.37  | 22.37| -14.87| 22    |
Table 3. *P*-values of three-way ANOVA for the effects of yield stability under drought (Ys), water treatment (W), stage (S) and their interactions on observed leaf traits of maize. Significant values (*P* < 0.05) are shown in bold. Trait abbreviations: A, net CO2 assimilation (µmol CO2 m$^{-2}$ s$^{-1}$); E, transpiration (mmol H2O m$^{-2}$ s$^{-1}$); gs, stomatal conductance (mol H2O m$^{-2}$ s$^{-1}$); Ci, intercellular CO2 concentration (µmol mol$^{-1}$); WUEi, instantaneous water use efficiency (µmol CO2 mmol$^{-1}$ H2O); WUEin, intrinsic water use efficiency (µmol CO2 mol$^{-1}$ H2O); Tl, temperature of adaxial leaf surface (°C); ChlSPAD, chlorophyll content; Ψw, leaf water potential (-MPa); Ψz, leaf osmotic potential (-MPa); Ψz100, leaf osmotic potential at full turgor (-MPa); RWC, relative water content (%); Suc, succulence (g g$^{-1}$); SLA, specific leaf area (cm$^{2}$ g$^{-1}$); TSD, total stomatal density (mm$^{-2}$).

| df  | A   | E   | gs  | Ci  | WUEi | WUEin | Tl  | ChlSPAD | Ψw  | Ψz  | Ψz100 | RWC | Suc  | SLA | TSD  |
|-----|-----|-----|-----|-----|------|-------|-----|---------|-----|-----|-------|-----|------|-----|------|
| Ys  | 1   | 0.141 | 0.349 | 0.393 | 0.652 | 0.384 | 0.999 | 0.449 | 0.090 | 0.124 | 0.118 | 0.780 | 0.023 | 0.008 | 0.590 |
| W   | 2   | <0.001 | <0.001 | <0.001 | 0.372 | 0.743 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.372 | 0.470 |
| S   | 1   | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.022 | 0.622 | 0.002 | <0.001 | <0.001 | <0.001 | <0.001 | 0.470 |
| Ys×W | 2   | 0.226 | 0.301 | 0.347 | 0.788 | 0.578 | 0.970 | 0.778 | 0.152 | 0.346 | 0.648 | 0.612 | 0.607 | 0.610 | 0.447 | 0.396 |
| Ys×S | 1   | 0.614 | 0.668 | 0.924 | 0.305 | 0.383 | 0.262 | 0.236 | 0.071 | 0.141 | 0.609 | 0.326 | 0.400 | 0.133 | 0.644 | 0.626 |
| W×S | 2   | <0.001 | 0.002 | <0.001 | <0.001 | <0.001 | <0.001 | 0.303 | 0.346 | 0.002 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Ys×W×S | 2   | 0.222 | 0.360 | 0.424 | 0.633 | 0.501 | 0.673 | 0.918 | <0.001 | 0.780 | 0.242 | 0.397 | 0.764 | 0.763 | 0.574 | 0.371 |
### Table 4

P-values of three-way ANOVA for the effects of genotype (G), water treatment (W), stage (S) and their interactions on observed leaf traits of maize. Significant values (P < 0.05) are shown in bold. Trait abbreviations: A, net CO₂ assimilation (µmol CO₂ m⁻² s⁻¹); E, transpiration (mmol H₂O m⁻² s⁻¹) gₛ, stomatal conductance (mol H₂O m⁻² s⁻¹); Cᵢ, intercellular CO₂ concentration (µmol mol⁻¹); WUEᵢ, instantaneous water use efficiency (µmol CO₂ mmol⁻¹ H₂O); WUEᵢᵣ, intrinsic water use efficiency (µmol CO₂ mol⁻¹ H₂O); Tₛ, temperature of adaxial leaf surface (°C); ChlSPADₚać, chlorophyll content; Ψₚ, leaf water potential (-MPa); Ψₛ, leaf osmotic potential (-MPa); Ψₛ₁₀₀, leaf osmotic potential at full turgor (-MPa); RWC, relative water content (%); Suc, succulence (g g⁻¹); SLA, specific leaf area (cm² g⁻¹); TSD, total stomatal density (mm⁻²).

| df | A  | E  | gₛ | Cᵢ | WUEᵢ | WUEᵢᵣ | Tₛ | ChlSPADₚać | Ψₚ | Ψₛ | Ψₛ₁₀₀ | RWC | Suc | SLA | TSD |
|----|----|----|----|----|------|-------|----|------------|----|----|------|-----|-----|-----|-----|
| G  | 5  | <0.001 | <0.001 | 0.553 | 0.455 | 0.562 | 0.794 | <0.001 | 0.157 | 0.003 | 0.431 | 0.026 | <0.001 | 0.001 | <0.001 |
| W  | 2  | <0.001 | <0.001 | <0.001 | <0.001 | 0.375 | 0.764 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| S  | 1  | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.009 | 0.761 | 0.002 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| G×W | 10 | 0.008 | 0.032 | 0.081 | 0.255 | 0.159 | 0.221 | 0.909 | 0.008 | 0.049 | 0.272 | 0.023 | 0.410 | 0.446 | 0.253 | 0.752 |
| G×S | 5  | 0.080 | 0.183 | 0.163 | 0.602 | 0.817 | 0.266 | 0.786 | 0.009 | 0.014 | 0.570 | 0.162 | 0.292 | 0.196 | 0.010 | 0.006 |
| W×S | 2  | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.359 | 0.209 | 0.001 | 0.208 | <0.001 | <0.001 | 0.631 | 0.274 | 0.122 | 0.122 |
| G×W×S | 10 | 0.065 | 0.187 | 0.326 | 0.302 | 0.452 | 0.046 | 0.992 | <0.001 | 0.071 | 0.428 | 0.657 | 0.980 | 0.149 | 0.322 | 0.842 |
FIGURE LEGENDS

**Figure 1.** Observed vs predicted cross-validated values of 14 key leaf traits for detection of drought in maize. Error bars for predicted values represent the standard deviations generated from the 500 simulated models. Dashed line is 1:1 relationship. Trait abbreviations: A, net CO2 assimilation (µmol CO2 m⁻² s⁻¹); E, transpiration (mmol H2O m⁻² s⁻¹) gₛ, stomatal conductance (mol H₂O m⁻² s⁻¹); Ci, intercellular CO2 concentration (µmol mol⁻¹); WUEₛ, instantaneous water use efficiency (µmol CO2 mmol⁻¹ H₂O); WUEᵢᵣ, intrinsic water use efficiency (µmol CO2 mol⁻¹ H₂O); Tₛ, temperature of adaxial leaf surface (°C); ChlSPAD, chlorophyll content; Ψₛ, leaf water potential (-MPa); Ψᵢ, leaf osmotic potential (-MPa); Ψᵢ₁₀₀, leaf osmotic potential at full turgor (-MPa); RWC, relative water content (%); Suc, succulence (g g⁻¹); SLA, specific leaf area (cm² g⁻¹); TSD, total stomatal density (mm⁻²).

**Figure 2.** Mean (solid), 5th and 95th percentile (dotted) of standardized coefficients (black) and variable importance for projection values (VIP, blue) by wavelengths for PLSR-models predicting 14 key leaf traits for detection of drought in maize. Trait abbreviations: A, net CO2 assimilation (µmol CO2 m⁻² s⁻¹); E, transpiration (mmol H2O m⁻² s⁻¹) gₛ, stomatal conductance (mol H₂O m⁻² s⁻¹); Ci, intercellular CO2 concentration (µmol mol⁻¹); WUEₛ, instantaneous water use efficiency (µmol CO2 mmol⁻¹ H₂O); WUEᵢᵣ, intrinsic water use efficiency (µmol CO2 mol⁻¹ H₂O); Tₛ, temperature of adaxial leaf surface (°C); ChlSPAD, chlorophyll content; Ψₛ, leaf water potential (-MPa); Ψᵢ, leaf osmotic potential (-MPa); Ψᵢ₁₀₀, leaf osmotic potential at full turgor (-MPa); RWC, relative water content (%); Suc, succulence (g g⁻¹); SLA, specific leaf area (cm² g⁻¹); TSD, total stomatal density (mm⁻²).
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