Detection of the Metabolic Response to Drought Stress Using Hyperspectral Reflectance

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Highlight
Drought stress responses, including a drought-signalling plant hormone, can be measured using light reflected from leaves before drought is visible to the naked eye.

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Abstract

Drought is the most important limitation on crop yield. Understanding and detecting drought stress in crops is vital for improving water use efficiency through effective breeding and management. Leaf reflectance spectroscopy offers a rapid, non-destructive alternative to traditional techniques for measuring plant traits involved in a drought response. We measured drought stress in six glasshouse-grown agronomic species using physiological, biochemical and spectral data. In contrast to physiological traits, leaf metabolite concentrations revealed drought stress before it was visible to the naked eye. We used full-spectrum leaf reflectance data to predict metabolite concentrations using partial least-squares regression, with validation $R^2$ values of 0.49 to 0.87. We show for the first time that spectroscopy may be used for the quantitative estimation of proline and abscisic acid, demonstrating the first use of hyperspectral data to detect a phytohormone. We used linear discriminant analysis and partial least squares discriminant analysis to differentiate between droughted and watered plants based on measured traits (accuracy: 71%) and raw spectral data (66%). Finally, we validated our glasshouse-developed models in an independent field trial. We demonstrate that spectroscopy can detect drought stress via underlying biochemical changes, before visual differences occur, representing a powerful advance for measuring limitations on yield.

Keywords

Abscisic Acid (ABA), Climate Change, Crop Breeding and Management, Drought Stress, Leaf Reflectance, Metabolites, Remote Sensing, Stress Responses, Water Deficit, Water Stress
Introduction

Water is vital for life. For much of the planet, climate change is creating a hotter, drier world, impacting plant productivity across both managed and unmanaged ecosystems (Franklin et al., 2016). Agricultural irrigation alone is responsible for two-thirds of our freshwater withdrawals (Poore & Nemecek, 2018), and the provision of water for agriculture is threatened by acute and chronic water shortages. These shortages are driven by increased drought – water shortage caused by low rainfall – and heightened demand for water for other purposes; both drivers will increase during the coming century (Food and Agriculture Organization of the United Nations; Cattivelli et al., 2008). At the same time, a significant increase in crop yield is required to feed the growing global population (Ort et al., 2015; FAO, 2018; Simkin et al., 2019). We therefore urgently need high-yielding crops with high water use efficiency, in addition to improved water management practices, in order to sustain future crop production (Morison et al., 2008; Leakey et al., 2019).

There are a number of plant traits indicative of a drought response. It is well known that plants respond to drought in myriad ways, underpinned by a suite of genetic, biochemical, and physiological adjustments and adaptations (Chaves et al., 2009; Fàbregas & Fernie, 2019). These responses are orchestrated by signalling pathways, mediated in particular by the phytohormone abscisic acid, ABA (Sreenivasulu et al., 2012; Munemasa et al., 2015; Sah et al., 2016; Zhu, 2016). ABA levels increase dramatically in drought-stressed leaves, and ABA regulates almost ten percent of protein-coding genes as well as inducing many genes involved in carbohydrate metabolism (Sreenivasulu et al., 2012; Thalmann & Santelia, 2017).
Physiologically, ABA decreases stomatal conductance in order to reduce water loss via transpiration, and this together with reduced rates of photosynthesis is a key indicator of drought stress (Chaves et al., 2009). Biochemically, species differ in their responses to drought but typically show an early increase in the content of leaf sugars, which can act as osmotic protectors (Thalmann & Santelia, 2017), followed by an increase in ABA and amino acids, including the important osmotic protector proline (Fàbregas & Fernie, 2019).

Characterising the drought stress responses of plants is essential for understanding the effects of drought, and the interactions of these effects with key life processes such as growth and reproduction. This is critical for developing effective tools for crop breeding and management in the face of reduced water supply, for example the capacity to screen different crop varieties for drought stress tolerance in order to select parents for breeding. Traditional techniques for measuring drought stress are extremely robust yet can be costly and time-consuming. Remote sensing techniques offer a novel solution to improve the speed of detection for small and large-scale applications (AghaKouchak et al., 2015). The physiological and metabolic profile of a drought-stressed plant is traditionally captured with measurements of photosynthetic parameters using gas exchange and fluorescence, and biochemical measurements following destructive leaf harvesting. Physiological breeding (Reynolds & Langridge, 2016) involves the consideration of physiological traits, including those related to water use efficiency, in crop breeding systems (Leakey et al., 2019). This increases the efficacy of breeding for abiotic stress resilience, yet lengthy measurements reduce throughput. Physiological breeding would be greatly enhanced by remote sensing techniques which deliver high-throughput, non-
destructive, and relatively low-cost estimates of critical physiological and biochemical plant
traits for crop breeding and management using passive optical and thermal imaging techniques
inter alia (Serbin et al., 2012, 2014, 2015; Couture et al., 2016; Yendrek et al., 2017; Silva-Perez
et al., 2018; Ely et al., 2019; Meacham-Hensold et al., 2019; Shiklomanov et al., 2019). In
particular the use of high spectral resolution spectroscopic reflectance data – the collection of
numerous contiguous reflected wavelengths – to predict drought phenotypes is of great value
for crop breeders screening for improved water use efficiency and drought stress tolerance,
and for farmers managing water supply for maximal yield in a changing environment. Crucially,
quantifying drought stress before it can be detected visually – by identifying plants in the pre-
drought condition using spectroscopy to detect known leaf-level metabolic changes caused by
drought stress – would provide a major advance in drought prediction and mitigation.

Spectral data can predict a range of biochemical and physiological traits, with several key
advances using partial least-squares regression (PLSR) to predict diverse traits of interest in
recent years. For example, models to predict maximum carboxylation capacity of Rubisco, and
electron transport rate, have been developed in aspen and cottonwood (Serbin et al., 2012),
maize (Yendrek et al., 2017), wheat (Silva-Perez et al., 2018), tobacco (Meacham-Hensold et al.,
2019) and crop canopies (Serbin et al., 2015). Prediction of a range of leaf structural and
biochemical traits has also been achieved, such as leaf mass per unit area (LMA) in a range of
tree species (Serbin et al., 2014), secondary metabolites such as tannins in birch and aspen
(Couture et al., 2016), chlorophyll in maize (Yendrek et al., 2017) and a suite of metabolites
including protein, starch, amino acids and several sugars in a range of agronomic species (Ely et
PLSR involves the building of a statistical relationship between spectroscopic data and measured traits. Subsequently, leaf traits may be predicted using this spectroscopic or “hyperspectral” data alone. Spectral data have also been used to examine drought stress in plants, but traditionally this has been performed using two-band spectral vegetation indices (SVIs) such as the normalised difference vegetation index (NDVI), photochemical reflectance index (PRI), and water band index (WBI) which are known to correlate with water stress and associated changes such as a change in pigment pools (Zarco-Tejada et al., 2012, 2013; Rossini et al., 2013; Ballester et al., 2018; Gerhards et al., 2019). However, these indices do not provide a mechanistic explanation: they do not reveal the metabolic and physiological processes underpinning the drought response. Furthermore, indices often do not show good generality between species and experiments (e.g. Rapaport et al., 2017).

Our approach moves beyond the use of indices correlated with drought, to detect specific metabolic signals linked to the mechanisms underpinning the drought response, using hyperspectral leaf reflectance data. We sought to use full-spectrum hyperspectral data, which is a more powerful and comprehensive measurement than an index using only two wavebands from within the spectrum, to predict the metabolic changes underpinning the drought response before it is visible to the naked eye in order to develop a rapid yet comprehensive picture of drought stress which is lacking when indices are used. Secondly, in addition to predicting changes in metabolites for which it has already been demonstrated that prediction is possible using hyperspectral data, we sought to use hyperspectral data to predict the concentrations of ABA and proline, which are two key metabolites in the drought response for which no
relationship with hyperspectral data has yet been developed. Indeed, to our knowledge no phytohormone has yet been predicted from hyperspectral data and this would greatly improve the prospects of using hyperspectral data for plant stress detection since phytohormones are frequently involved in stress signalling upstream of (and therefore earlier than) metabolic changes. Finally, we wished to examine the applicability of our approach by scaling up from the glasshouse environment to an independent field trial, carried out the following year.

In our glasshouse experiment, we imposed drought (a full dry-down treatment) on a diverse range of agronomic species and utilised physiological, biochemical and spectroscopic techniques to characterise the drought response. Rather than simulate a realistic, ephemeral drought stress using careful water monitoring, our aim was to generate a strong drought response in all species, thus the full dry-down method was selected (Fàbregas & Fernie, 2019). We hypothesised that spectroscopy could be used to quantitatively detect the critical indicators which are known to underpin the metabolic response to drought stress, including drought-induced changes in ABA and proline concentrations as well as carbohydrates, for the purposes of rapid and early identification of drought. To date, ABA and proline have not been predicted from spectral data, and this would provide a major advance to the field. Our overarching goal was to elicit a drought response and evaluate our ability to detect signals of drought stress using spectroscopy. Specifically, we addressed the following research questions: 1) Can metabolic signals be used to predict drought stress before drought is visible to the naked eye? 2) Can spectroscopy be used to detect these key metabolic signals? 3) Can spectroscopy be used to identify drought stress before signs of drought are detected visually?
Materials and Methods

Plant material and growth conditions for the glasshouse experiment

A range of agronomic species with differing morphological and physiological characteristics were selected, to maximise trait variation. As well as considering a range of characteristics to maximise trait variation, species were also selected based on the following two criteria. Firstly, each species has leaves that can readily be measured using a spectroradiometer, i.e. large enough to obtain quality measurements without the need for joining many samples together in a “raft”. Secondly, each species is readily grown in the Long Island region so that our findings are relevant for the local agricultural environment and to facilitate our subsequent field study which focused on one species of particular interest (C. pepo) identified from the results of the glasshouse study in order to validate our findings. Seeds of Capsicum annuum var. Sweet Chocolate (bell pepper), Cucurbita pepo L. var. Costata Romanesco (courgette), Helianthus annuus var. Pro Cut Gold F1 (sunflower) and Raphanus sativus var. Easter Egg (radish) were all obtained from Johnny’s Selected Seeds, Winslow, Maine, USA. Setaria italica (foxtail millet) and Sorghum bicolor (sorghum, mixed colors broomcorn) seeds were obtained respectively from Deer Creek Seed, Windsor, Wisconsin, USA and Seed Savers, Decorah, Iowa, USA. Populus x canadensis clone OP367 (hybrid poplar) cuttings were obtained from Cold Stream Farm, Free Soil, Michigan, USA. Plants were grown in the glasshouse at Brookhaven National Laboratory, Upton, New York, USA in spring and summer 2018 under a 14-hour photoperiod and temperatures of 17°C–33°C:17°C–29°C day:night. Plants were grown under ambient light conditions with supplementary lighting of 49 W m⁻² provided by high pressure sodium bulbs.
The maximum light level was 2078 μmol m\(^{-2}\) s\(^{-1}\) photosynthetic photon flux density (PPFD). The maximum value of relative humidity was 99%. Environmental conditions were recorded by a weather station located in the glasshouse. The station was comprised of a humidity and temperature sensor (ATMOS 14, METER Group, Inc., Pullman, WA, USA) and two quantum sensors (QSO S and PYR, Apogee Instruments, Logan UT, USA). Seeds were sown in BM2 germinating mix (Berger, Saint-Modeste, Quebec, Canada) and seedlings were subsequently transplanted to 30 litre pots filled with Pro-Mix BX Mycorrhizae growing medium (Premier Tech Horticulture, Quakertown, Pennsylvania, USA). Poplar cuttings were soaked in water until roots developed, then grown in 100 litre pots filled with 52 Mix (Conrad Fafard, Inc., Agawam, Massachusetts, USA). Plants were watered three times per week, with the exception of plants undergoing the drought treatment. Pots were raised to provide an air gap between the base of the pot and the glasshouse floor and prevent root exploration and water uptake from the glasshouse floor during the drought treatment. Osmocote Plus slow-release fertilizer (The Scotts Company, Marysville, Ohio, USA) was added according to manufacturers’ instructions, and pesticides were applied at fortnightly intervals in accordance with usage directions. Due to a low sample size, *S. italic* was omitted from the detailed species-level analysis but included in the modelling approaches to maximise trait diversity and increase robustness.

**Drought treatment and measurement schedule for glasshouse-grown plants**

Plants were randomly assigned to either the drought or the control treatment, with 50% of the plants of each species allocated to each treatment and distributed randomly. Water was completely withheld from droughted plants, from the onset of the drought treatment until the
end of the experiment, with the first day of missed watering being designated day 0 of the
drought treatment; control plants received water three times per week. For each species, the
experiment ended when leaves were fully desiccated, i.e. meaningful gas exchange, metabolic
or spectral data could no longer be collected. Final measurements were made for each species
as follows: after 35 days of drought for *C. annuum*, after 32 days for *C. pepo*, after 29 days for
*H. annuus*, after 37 days for *P. canadensis*, after 33 days for *R. sativus*, and after 23 days for *S.
bicolor*. During the drought treatment, soil moisture content was measured daily at 16:00 (HS2-
12 soil-water sensor, Campbell Scientific, Logan, Utah, USA). The 20cm probe was used for 100
litre pots and the 12cm probe was used for 30 litre pots. Spectral and gas exchange
measurements were performed between 08:30 and 13:30 at regular intervals throughout the
treatment. Leaf harvesting was carried out between 11:30 and 14:30, always paired with
collection of spectral data. For the main (glasshouse) experiment, \( n = 12 \) plants per treatment
for *C. annuum* and *C. pepo*; \( n = 8 \) plants per treatment for *H. annuus*, *R. sativus*, *P. canadensis*
and *S. bicolor*. At each time point in the experiment, one leaf was removed from each individual
and then discs were removed as described below. Each individual oven-dried or flash-frozen
sample was a pool of multiple discs from the same individual leaf, such that there were either
12 or 8 samples taken for each treatment at each time point (depending on the species)
representing true biological units (one sample per plant, with the experimental treatment
applied at the plant level). At each time point throughout the measurement period, a different
leaf was harvested from the same individuals. Spectral data were collected on more dates than
harvests were carried out, due to the destructive nature of leaf sampling. All measurements
were performed on the newest fully-expanded leaf. The leaf plastochron index (LPI) system
(Erickson & Michelini, 1957) was used to track leaf development over time in *P. canadensis*.

Measurements of *P. canadensis* focused on the leaf at position LPI 11, where LPI 0 was the first leaf to be at least 25mm long.

**Visual assessment of drought**

Plants were regularly photographed and the images visually assessed to establish the date on which a drought effect was first visible for each species in the aboveground part of the plant. To avoid bias this visual assessment was carried out in isolation once all photographs had been collected, and prior to all analysis of the physiological, biochemical and spectral data including any analysis of the number of days taken for drought signals to be identified using those techniques. Visible signs of drought varied between species (Fig. 1). In the first instance, visible signs were: wilting including a change in leaf angle in *C. annuum*, yellowing and wilting in *C. pepo*, wilting including a change in leaf angle in *H. annuus*, yellowing of the lower leaves in *P. canadensis*, wilting in *R. sativus*, and wilting including a change in leaf angle in *S. bicolor* (Fig. 1; Supporting Information Fig. S1).

**Gas exchange**

Survey-style gas exchange measurements were performed as described previously (Burnett *et al.*, 2019), for three species, using four LI-6400XT Portable Photosynthesis Systems, each with a Leaf Chamber Fluorometer (LI-COR, Lincoln, Nebraska, USA), zeroed using a common nitrogen standard. Measurements were taken following a brief (<2 min) stabilization period in the leaf cuvette. The reference CO₂ concentration was 400 μmol mol⁻¹. Saturating irradiances used for
gas exchange measurements were determined for each species using light response curves: 1,500 μmol photons m$^{-2}$ s$^{-1}$ for *R. sativus*; 1,800 μmol photons m$^{-2}$ s$^{-1}$ for *H. annuus* and *S. bicolor*. The flow rate was 200–500 μmol s$^{-1}$. Block temperature was set to ambient. Gas exchange data in the manuscript are presented in accordance with the data standard published by Ely *et al.* (2021). Raw gas exchange data are available in the Supplementary Dataset.

**Leaf traits**

Leaf samples were harvested using a metal punch of known area and were either immediately flash-frozen in liquid nitrogen for biochemical analysis, or oven-dried for elemental carbon and nitrogen analysis (oven-dried samples comprised a separate batch of material taken from the same leaf used for the flash-frozen samples). The selection of the leaf punch depended on the size of the leaf being harvested, and ranged from 0.95 to 3.46 cm$^2$; multiple discs were harvested for each sample and the total area calculated. Discs were taken from across the area of the leaf in order to account for the variation within a leaf. Leaf metabolic traits were then expressed on an area basis (see Supplementary Dataset). For each assay, samples were randomised prior to analysis. Analysis of elemental carbon and nitrogen on ground oven-dried samples was performed as described previously (Burnett *et al.*, 2016). Sufficient material was available to provide three aliquots of the frozen ground homogenised material of each sample, i.e. of the material from each harvested leaf. One aliquot of frozen material underwent ethanol extraction, followed by analysis of a suite of carbon- and nitrogen-containing metabolites (Burnett *et al.*, 2016, 2018), including fructans for monocot species. A second aliquot of frozen leaf material was used for the ABA assay. Samples were placed in 500μL ABA extraction buffer
per 33mg sample and incubated overnight on a shaker (4°C, 180rpm, in the dark). ABA extraction buffer contained 80% methanol (v/v in H₂O), 100mg/L butylated hydroxytoluene dissolved in methanol, and 500mg/L citric acid dissolved in H₂O. ABA assays were performed using Phytodetek assay kits using the competitive antibody binding method (Agdia, Elkhart, Indiana, USA) following an established method (Kim et al., 2012). The protocol was recovery-tested prior to use and ABA concentrations were determined using a standard curve. A third aliquot of frozen leaf material was used for the proline assay. Proline assays were performed following sample extraction in sulfosalicylic acid, acidic ninhydrin, and toluene (Sunkar, 2010); total free proline concentrations were determined using a standard curve. For every assay, two technical replicates were run for the material from each leaf and the results averaged to give one value per leaf sample.

Leaf water content (LWC) was measured using the following equation:

\[
LWC \, (\%) = \left( \frac{\text{leaf fresh mass} - \text{leaf dry mass}}{\text{leaf fresh mass}} \right) \times 100
\]

Leaf trait data, including metabolic traits and LWC, are available in the Supplementary Dataset.

**Spectroscopy**

Leaf spectral reflectance data were collected using a PSR+ full-range (continuous 350–2500nm) spectroradiometer (Spectral Evolution, Lawrence, Massachusetts, USA) connected to a leaf clip assembly with an internal, calibrated light source (SVC, Poughkeepsie, New York, USA). The spectroradiometer was calibrated using a LabSphere Spectralon® reflectance standard (LabSphere, Inc., North Sutton, New Hampshire, USA). 3-4 spectra were collected from across the adaxial surface of each leaf and averaged to give a single spectrum for each leaf at each
time point. Leaf temperature was measured immediately prior to each spectral measurement using an infrared radiometer (Apogee Instruments, Logan, Utah, USA). Spectral data collection was performed according to established procedures (Serbin et al., 2012; Ely et al., 2019). Spectral data are available in the Supplementary Dataset.

Field-grown C. pepo used for validation of the approach

Seeds of C. pepo L. var. Dunja were obtained from the Long Island Cauliflower Association (Riverhead, New York, USA) and sown in twelve 10m x 10m plots, surrounded by a C. pepo border the same width as 1.5 times the height of the mature vegetation and sown at a density to achieve full canopy coverage, in a research field at Brookhaven National Laboratory, Upton, New York, USA in 2019. Following germination and establishment, irrigation was maintained at standard local agricultural levels for watered plants (six plots), and withheld for droughted plants (six plots). Biochemical and spectral data were collected on these plants as described above, within three hours of solar noon, with n=3 reps per plot on each measurement day. Each replicate of leaf material was composed of a set of leaf discs punched from across the area of the leaf and pooled to make one sample from one leaf, thus material from three plants per plot was harvested on each measurement day. Since the experimental treatment was applied at the plot level, the three plants per plot represent pseudoreplicates, with n=6 droughted plots and 6 watered plots measured on each day. Due to the abundance of plant material, different plants within each plot were measured on each measurement day. Each leaf was harvested immediately following spectral data collection.
Data analysis was performed in the R open source software environment (R Core Team, 2019). For statistical analysis of physiological traits, an overall linear mixed-effects model was first fit for each trait for each species in turn, testing for effects of (and interactions between) treatment categories and time into the drought experiment, keeping the individual plant as a random effect. Subsequently, to examine differences between watered and droughted plants at individual time points, repeated-measures ANOVA was performed for each trait for each species to account for the repeated nature of the measurements performed on individual plants outlined above. Then, estimated marginal means were calculated and a post-hoc Tukey test was performed to identify non-cumulative, pairwise differences between droughted and watered plants at each individual time point. For statistical analysis of leaf biochemical traits (measured using traditional approaches), an initial linear mixed-effects model was first fit for all data for each trait to test for effects of (and interactions between) the fixed effects of species, treatment and time into the drought experiment, keeping the individual plant as a random effect. Then, a repeated-measures ANOVA with a post-hoc Tukey test was performed for each species and each trait, to compare each time point, as described above. When required, data were log-transformed prior to analysis to provide normality.

We developed relationships between spectral data and leaf traits measured on an area basis using a partial least-squares regression (PLSR) modelling approach. PLSR was performed using the ‘pls’ package (Mevik & Wehrens, 2007) in R, following methods developed previously (Serbin et al., 2014; Ely et al., 2019). PLSR models perform best when a wide range of trait
values are used to build the model (i.e. the use of a large ‘trait space’), in order to establish strong predictive power within that range of values (Schweiger, 2020). For this reason, for all metabolites in this study, except ABA, data from all species were pooled for model training and testing. For ABA, the PLSR model using the full-species dataset only yielded an $R^2$ value of 0.09; thus it was decided to build an ABA PLSR model using data from C. pepo only which yielded a much higher $R^2$.

For PLSR model building, spectral wavelengths of 500–2400 nm were used for all trait predictions with the exception of starch and the C. pepo-only model for ABA. In these two cases the range 1100–2400 nm was used to improve the accuracy of prediction. For two models, proline and the C. pepo-only model for ABA, spectral data were transformed prior to analysis, using $\log_{10}(1/\text{reflectance})$, to improve model fit. When appropriate, leaf trait data were $\log_{10}$- or square-root-transformed in order to produce a normal distribution prior to PLSR as detailed in Table 1, and a small number of samples were rejected from the dataset due to outlier residual errors in order to develop a model reflecting the trait space covered by the majority of data points. Observational data points were subset according to species then randomly assigned to calibration and validation datasets composed of 80% and 20% of the data, respectively. Component selection and model calibration were carried out as described previously (Serbin et al., 2014; Ely et al., 2019). The $R^2$ and root mean square error (RMSE) of prediction of the validation data set was used to assess each model. The variable importance of projection (VIP) was used for qualitative evaluation of model predictor variables (Wold et al., 2001). For the
application of glasshouse-built PLSR models to novel spectra collected on field-grown *C. pepo*, glasshouse-derived model coefficients were applied to field spectra for trait prediction.

Discriminant analyses were used to identify the ability to statistically separate control and drought treatment classes in our datasets. Linear discriminant analysis (LDA), was for use with our measured or predicted trait data and partial least squares discriminant analysis (PLS-DA) was for use with spectral data. The LDA model built with glasshouse data used a 75:25 calibration/training and model validation/testing split of the data stratified according to treatment; model training was performed with leave-one-out calibration. Receiver-operator curve (ROC)-optimisation of the number of components was performed; ROC analysis and the standard area under the curve (AUC) value indicating model accuracy of prediction for the glasshouse LDA model are presented in Supporting Information Fig. S2. LDA models for field data were built with leave-one-out calibration, and were based on the suite of traits established and validated for the glasshouse models, taking into account the effectiveness of trait prediction in the field.

PLS-DA models built for the main (glasshouse) dataset also used a 75:25 split of the data for model training and model testing, with 10-fold cross-validated resampling repeated five times on the training data, and ROC-optimisation of the number of components. ROC analysis and AUC values are presented in Supporting Information Fig. S3. PLS-DA models ran for up to 100 iterations, until convergence was reached. When PLS-DA models were applied to spectral data for field-grown *C. pepo*, the split of model training (glasshouse) and testing (field) data deviated
from 75:25, as detailed in Table 2. In each case the full glasshouse dataset was used for model calibration with field data used as a validation of model performance in the outdoor setting. For the full-species models for metabolite data, PLS-DA, PLSR and LDA, *S. italic*ca data were included in order to increase the trait variation and therefore the predictive capability of the models; *S. italic*ca was excluded from species-level metabolite plots due to low sample size. PLS-DA was performed using the ‘caret’ package (Kuhn, 2008) in R, as described previously (Cotrozzi & Couture, 2020; Gold *et al.*, 2020). For our PLS-DA models we also report the number of components used for each permutation of the data (Table 2). The number of components is an essential attribute, and is sometimes provided (Cavender-Bares *et al.*, 2016; Fallon *et al.*, 2020), yet many other past studies have often omitted to report the associated number of PLS-DA components (e.g. Susič *et al.*, 2018; Zovko *et al.*, 2019). We openly report our data and encourage other authors to do the same to facilitate more fair comparisons of overall model performance.
Results

Physiological measurements only reveal drought after visual drought detection

Visual detection of drought occurred on different days for the six different species in the main study, ranging from 8 to 24 days into the treatment (Fig. 1, Supporting Information Fig. S1). Gas exchange measurements were performed for three species and revealed a significant and marked decrease in net CO₂ exchange per leaf area ($A$) and stomatal conductance to water vapor per leaf area ($g_{sw}$) in droughted compared to watered plants of each species (Fig. 2). *H. annuus* (Fig. 2a,d) maintained higher rates of $A$ and $g_{sw}$ throughout drought when compared with *S. bicolor* (Fig. 2c,f) in which we observed near-zero rates of $A$ and $g_{sw}$ at the end of the drought treatment. This is consistent with the idea that *H. annuus* is anisohydric, meaning that it maintains water use and $A$ during drought at the possible cost of hydraulic failure, while *S. bicolor* is known to be isohydric, limiting $g_{sw}$ as a method of reducing risk of hydraulic failure while reducing $A$ and risking carbon starvation. Differences between $A$ and $g_{sw}$ in watered and droughted *H. annuus* occurred later into the drought treatment and were of proportionally smaller magnitude than the effect observed in *S. bicolor* (Fig. 2a,c,d,f). Notably, the decrease in $A$ and $g_{sw}$ always occurred after visual detection of drought in each species (Fig. 2). Therefore, for the species we investigated here, traditional measurements of leaf gas exchange are inadequate for early detection of drought stress since changes in photosynthesis and stomatal conductance only manifested after visible drought effects were observed.
Biochemistry reveals pre-visual metabolic indicators of drought

The metabolic profile of each species varied greatly, with different sets of metabolites indicating a response to drought stress (Fig. 3). Broadly speaking, the drought indicators ABA and proline increased during drought along with elemental leaf nitrogen, protein and total free amino acids whilst starch decreased (see Fig. 3 legend). In addition to the different metabolic profiles, the magnitude of the drought stress response varied greatly between species, with C. pepo and P. canadensis showing the greatest percent metabolic changes between droughted and watered plants. Thirdly, the timing of the response differed between species, with pre-visual changes in metabolite levels occurring in four of the six species. These pre-visual changes were strongest in C. annuum and C. pepo: each showed a sustained change in one or more metabolites prior to visual drought detection (Fig. 4). ‘Sustained’ indicates that the change was significant from its onset until the end of the drought experiment.

The key pre-visual, sustained indicator of drought stress was ABA in both C. annuum and C. pepo, accompanied by starch in C. annuum. In C. annuum, starch decreased (P < 0.05 at 21 days into drought treatment, subsequently P < 0.001; increase at first two time points was non-significant) and ABA increased throughout the drought treatment, significant from 21 days into drought treatment onwards (P < 0.05, subsequently P < 0.01; the magnitude of the relative difference varied over time). For both starch and ABA, the first significant differences were detected three days before visual detection of drought in C. annuum. In C. pepo, a significant increase in ABA (P < 0.01, subsequently P < 0.001) was detected from six days into the drought treatment, providing an early indication of drought five days before visual detection. The
relative difference in ABA between watered and droughted plants persisted throughout the experiment, with an initial spike followed by differences of steadily increasing magnitude as time progressed. In addition to these sustained changes, both C. annuum and C. pepo displayed an unsustained but pre-visual significant increase in protein (P < 0.05 in each case; initial decrease in protein seen in Fig. 3 non-significant for either species). Finally, H. annuus displayed a short-term pre-visual increase in nitrogen (P < 0.05) while P. canadensis displayed an unsustained pre-visual change in total non-structural carbohydrates (the sum of glucose, fructose, sucrose and starch) and glucose (P < 0.05 in each case).

Later in the drought treatment, once drought was already visible to the naked eye, we observed a significant difference in each of the six metabolites for C. annuum and C. pepo, as characterised in Fig. 3. Considering these six metabolites, in H. annuus a significant difference was observed for nitrogen, ABA and amino acids; in P. canadensis for starch, protein, ABA and amino acids; in R. sativus for all six metabolites except protein; and in S. bicolor for starch, ABA, amino acids and proline. Thus, ABA accumulates in droughted plants of all species and proline accumulates in droughted plants of C. annuum, C. pepo, R. sativus and S. bicolor. Due to the large changes observed in droughted plants for each trait (Fig. 3), we next hypothesised that the combination of these six metabolites would be sufficient to predict the droughted condition of plants, due to additive effects of small non-significant pre-visual changes combining with significant effects to enable early detection of drought in all species.
Leaf water content (LWC) was maintained until late into the drought treatment, and did not show a statistically significant change until after the time at which drought could be detected with the naked eye, in each species studied. In *C. annuum*, LWC did not change in droughted plants until the final time point, 2 days after drought was detected with the naked eye. At this time, LWC was lower in droughted plants (*P* < 0.001). In *C. pepo*, LWC showed a significant difference (*P* < 0.01) 27 days into the drought treatment, which was 16 days after drought was visible to the naked eye, yet there was no difference in LWC in subsequent measurements. In *H. annuum*, LWC never showed a significant difference between droughted and watered plants.

Plants of *P. canadensis* showed a significant difference (*P* < 0.05) in LWC 15 days after drought was visibly detectable, but as observed for *C. pepo*, this difference did not persist in later measurements. *R. sativus* plants showed a decrease (*P* < 0.001) in LWC in droughted plants at the final time point, 15 days after visual detection of drought. Finally, *S. bicolor* showed a decrease in LWC (*P* < 0.05, increasing to *P* < 0.001) in droughted plants, starting 6 days after visual detection of drought.

**Linear discriminant analysis allows prediction of drought from key metabolites**

We performed a linear discriminant analysis (LDA) with measured values of the six key leaf traits associated with the observed drought response: nitrogen, starch, protein, ABA, amino acids and proline. Data were separated for model building (*n* = 401) and model validation (*n* = 133). The success of prediction of the class ‘droughted’ or ‘watered’ for the validation dataset, averaged across ten iterations, was 71% (+ 4% standard deviation) with a kappa value of 0.42 (+ 0.08 standard deviation) and an AUC of 0.77 (+ 0.04 standard deviation; see Supporting
Information Fig. S2). With reference to the overall model including all time points, success of prediction increased when progressively later time points were selected and earlier time points excluded, since drought effects were more pronounced towards the end of the experiment making classification easier.

We have demonstrated that the robust approach of measuring metabolites using traditional biochemical techniques facilitates early detection of drought stress (research question 1), shown by analysing individual traits and developing an all-species LDA model for drought prediction. However, the destructive nature of these measurements and the relatively time-consuming process of laboratory analysis are two major disadvantages of using this technique for rapid detection of drought stress in the breeding or production environment. Therefore, we next examined whether spectroscopy could be used to characterise the drought-related metabolic profiles of plants (2), and whether this technique could also be used for pre-visual detection of drought stress (3).

Spectroscopy enables rapid accurate prediction of leaf metabolites

Partial least-squares regression (PLSR) models were used to build relationships between observed leaf traits derived from traditional biochemical techniques, and traits predicted using spectroscopic data (Fig. 5). Spectroscopic data are both non-destructive and quick to collect, overcoming the two disadvantages associated with collecting and analysing biochemical data identified above. Independent validation of PLSR for key drought-related traits reveals a strong capacity for the prediction of nitrogen, starch, protein, ABA, amino acids and proline (Fig. 5).
For the first time we developed relationships between biochemically-observed and spectrally-predicted values of ABA and proline with validation $R^2$ values of 0.50 and 0.84 respectively (Fig. 5d,f). This suggests it may be possible to use spectral data to estimate these key drought indicators non-destructively.

Spectroscopy enables early detection of drought

Having demonstrated the use of metabolic drought signals to detect drought pre-visually using biochemical analysis, and used spectral data to reveal those signals rapidly and non-destructively using PLSR models, we turned to our third question: can spectroscopy be used to identify drought stress before visual detection? We evaluated two approaches: the use of partial least squares discriminant analysis (PLS-DA) to interrogate the spectral data directly, and the use of PLSR to predict traits that were then used to detect drought stress using a standard LDA approach. Each approach was developed and tested using glasshouse data. Finally, the applicability and robustness of each approach was tested by independent validation using spectra collected from a separate drought experiment conducted in the field.

PLS-DA enables accurate drought classification from spectral data

Firstly, PLS-DA was used to determine the treatment class of leaf samples (‘droughted’ or ‘watered’) using full-range spectra from all data points in the study with an overall validation accuracy of 66% (Table 2). When all-species data were divided temporally into quartiles, classification accuracy increased with each successive quartile due to the increasing severity of the treatment (Table 2). To test the robustness of this diagnostic approach, PLS-DA models built
using the data from the main experiment performed in the glasshouse were applied to spectra measured on *C. pepo* grown in the field in a separate drought experiment (Table 2). When the main all-species dataset was used as the PLS-DA model training data, with novel field-grown *C. pepo* data as the testing data, the success of droughted or watered class prediction for field-grown *C. pepo* was 63%. When the testing data were composed of field-grown *C. pepo* spectra from only the second half of the drought treatment period rather than the entire period, overall model accuracy decreased to 53% (Table 2) due to mitigation of the drought treatment by rainfall events. However, when the testing data were composed of only the field-grown *C. pepo* spectra from the first half of the drought treatment period rather than the entire period, the model predicted the class of field-grown *C. pepo* with 71% accuracy (Table 2) indicating good detection of the onset of drought stress in the field – the most critical time for stress detection.

**Predicted traits derived from spectra enable accurate classification of drought**

Secondly, PLSR-derived predicted traits were used to examine the possibility of detecting the droughted or watered class. PLSR models developed from the main experiment (Fig. 5) were applied to spectra collected from the independent field-grown *C. pepo* to generate predictions of the key metabolites identified in Fig. 3. The predicted values of each trait obtained using these glasshouse PLSR models were then validated against observed metabolite data from the field experiment (Fig. 6). Extrapolating models from the glasshouse to the field is notoriously difficult because glasshouse- and field-grown plants have different leaf structures due to varying abiotic and biotic conditions (Poorter *et al.*, 2012, 2016). Despite these challenges, trait
prediction was successful for both starch and protein (Fig. 6a, b) and moderate for ABA and amino acids (Fig. 6c, d). When applying our glasshouse models to the field, however, trait prediction for proline was unsuccessful (Fig. 6e). The effective estimation of proline from spectral data in the glasshouse (Fig. 5f) indicates that this metabolite is especially sensitive to issues of model tuning between the glasshouse and field. We expect that re-tuning models with field data would yield a more widely applicable model.

LDA was performed using predicted trait values for starch, protein, ABA, nitrogen and amino acids, obtained from PLSR modelling performed on field spectra. These predicted traits showed moderate success at predicting the droughted or watered class of C. pepo field samples with 63% accuracy (n=321). When the five observed field traits (observed starch, protein, ABA, amino acids and proline) obtained from biochemical measurement of field samples were used in LDA the accuracy was 67% (n=182). When predicted field traits for only the first ten days of the drought treatment were used in LDA, class prediction accuracy was 74% (n=70). Thus, the predicted traits achieved a moderate level of class prediction accuracy exceeding the prediction accuracy of observed traits. This is likely due to marked qualitative differences in the magnitude of traits between droughted and watered plants, i.e. a qualitative “low” or “high” trait value may be predicted using PLSR even whilst the quantitative predicted trait value deviates from the observed value; the error in the PLSR models is normal meaning that additional bias is not introduced. The class prediction accuracy from predicted traits was highest (74%) during the first ten days of the drought treatment in the field indicating its potential for early detection of drought stress.
Discussion

Traditional approaches used to quantify the impacts of drought stress on crop physiology include measurements of gas exchange and fluorescence. While providing detailed information, these measurements are often slow and laborious, limiting their utility for field trials and rapid phenotyping (Rapaport et al., 2017; Burnett et al. 2019). Furthermore, as we have shown here, metabolic changes occur in advance of physiological changes and these key signals are even more difficult to analyse in real-time and require destructive harvesting, preventing their effective use in crop management. New approaches that can enable rapid non-destructive monitoring of crop status are critically needed to advance plant breeding for the production of food, fuel and fibre in a future production environment where drought stress will be more common. Integrating different methods will be important to enable the necessary advances (Cattivelli et al., 2008). Spectroscopy has already been successfully used to predict biochemical and physiological traits in crop plants (Yendrek et al., 2017; Silva-Perez et al., 2018). Here we have demonstrated that leaf metabolic indicators can be used to predict drought stress before it is visible to the naked eye (research question 1), that reflectance spectroscopy can be used to detect these key metabolic indicators of drought (2), and that this technique can be used to detect drought before it becomes visually apparent (3). We have shown for the first time that ABA and proline – two crucial players in the drought response – can be predicted from leaf reflectance spectroscopy. We have also shown, for the first time, that spectroscopy can be used to predict drought stress via detection of the underlying metabolic changes. Taken together, these novel results highlight the use of spectroscopy as a valuable approach yielding new, high-throughput, metabolite-focused tools for crop breeding and management.
High-throughput phenotyping is a vital part of crop breeding (Claeys & Inzé, 2013; Araus et al., 2018) and remote sensing will make an important contribution (Araus & Cairns, 2014). We present two avenues for achieving pre-visual detection of drought stress using spectroscopy: the use of PLS-DA to detect drought directly from hyperspectral data based on a trained model, and the use of PLSR-derived predicted metabolic traits to detect drought through a trait-focused LDA (Fig. 5, Fig. 6, Fig. 7), that allows for the elucidation of known response mechanisms in plants. The use of a trait-based approach requires the initial development of PLSR models using observed biochemical data for traits of interest, yet has the advantage of bringing specific predictions about trait values which may be of great importance in a plant breeding context. Moreover, once the model is developed, estimation of these traits in new leaves only requires the measurement of leaf reflectance (e.g. Serbin et al., 2019), meaning this approach provides a means for rapid trait evaluation in plants. The PLS-DA approach using raw spectra requires no initial biochemical data collection leading to an increased speed and reduced cost, but whilst more comprehensive than a two-band spectral index it inherently provides less information about the metabolic status of the plants and requires a training dataset of spectra associated with known treatments.

Remote sensing techniques have been used to examine drought stress in the past, providing the advantage of being rapid, high-throughput and non-destructive compared to traditional physiological and biochemical tools for measuring drought. However, drought stress detection using remote sensing has typically focused on spectral indices (Behmann et al. 2014; Becker and
Computational approaches have also been used to analyse the full-spectrum data of hyperspectral images, expanding upon the utility of indices, yet without employing a mechanistic or trait-based approach (Römer et al., 2012; Asaari et al., 2019). Our study moves beyond the use of more traditional spectral vegetation indices to detect water status to provide quantitative links between spectral signatures and the underlying physiological changes that alter leaf reflectance (e.g. Fig. 5b). It also builds on and advances the demonstrated use of hyperspectral data for pre-visual detection of drought stress (Bayat et al., 2016) and the quantitative relationships between spectra and leaf traits in crop plants (Yendrek et al., 2017; Silva-Perez et al., 2018; Ely et al., 2019). Here we expand upon these previous examples by allowing for the attribution of the drought response to its underlying physiological traits, facilitating pre-visual detection of drought stress by predicting the values of known traits which change within the leaf before drought can be seen using the naked eye. This enables the integration of different approaches to provide greater insight into the drought response. Identifying key metabolic indicators of drought stress in the first instance but then applying spectroscopy techniques to predict these indicators throughout a crop breeding programme or growing season offers a much more efficient system for pre-visual and real-time measurement of plant stress. Moreover, in contrast to biochemical data reliant upon harvests followed by post hoc analysis, spectral data can be collected and analysed in a continuous manner throughout an experiment, further increasing the power of this technique for detecting an effect.
Although physiological changes form an important and well-known part of the drought response, in the present study we did not observe a physiological change before drought was visible to the naked eye. The isohydric and anisohydric strategies of different plants – i.e., whether plants maintain water potential or tolerate a decrease in water potential under drought stress – form an additional consideration when examining stress responses (Tardieu et al. 1996; Tardieu and Simonneau, 1998; McDowell et al. 2008). Plants which tolerate a decrease in water potential, such as H. annuus, show a smaller decrease in photosynthesis under drought conditions making gas exchange measurements a less effective means of detecting drought stress than in isohydric species, demonstrated in our study by the maintenance of photosynthesis in H. annuus up until the end of the experiment (Fig. 2), although we found that no species showed a pre-visual change in either \( A \) or \( g_{sw} \).

In contrast to our physiological data, biochemical data revealed significant differences in advance of visual detection of drought (Fig. 7a), which is a key novel finding from this study. We observed an increase in ABA in all six species and an increase in proline in four species under drought conditions (Fig. 3) whilst sugars (glucose, fructose and sucrose) were not a major driver of the drought response in our study. Leaf starch often decreases under drought stress, in line with lowered photosynthetic capacity (Thalmann & Santelia, 2017), and this was the case for all six crops examined here (Fig. 3). The novel relationships we have shown between observed and predicted levels of ABA and proline (Fig. 5d,f) demonstrate an exciting potential for the non-destructive estimation of key drought stress metabolites, which we believe may be extrapolated to further phytohormones and signalling molecules that are central, early
components of plant responses to a wide range of abiotic and biotic stresses (Claeys & Inzé, 2013).

For most traits, our PLSR models showed high accuracy and low error (Fig. 5). However, when comparing the models, the performance of the ABA model was lower with an RMSE 19% of the observed data range for ABA, compared to RMSEs between 7% and 14% of the observed data range for the other traits (Fig. 5). Despite this, we believe that our findings represent a promising potential tool for drought detection for the suite of traits presented here. In particular, further research and additional datasets will likely improve the generality of the models (e.g. Serbin et al., 2019), including for ABA, particularly if a broader data range can be including in the model training. Furthermore, it should be noted that the validation plots presented in Fig. 5 are the results of an independent PLSR model validation, which provides a more accurate yet more conservative indication of model performance compared to the cross-validation approach used in many other studies (see Serbin et al. 2014, Table 4).

Our PLS-DA model results are comparable to those of previous studies which have demonstrated predictive capabilities with approximately 20 components in several models (Cavender-Bares et al., 2016; Fallon et al., 2020) and in one instance 34 components for a prediction accuracy of 61% (Fallon et al., 2020). Although metabolic profiles under drought stress varied between the different species in our study, and the performance of the PLSR models differed between traits, the overall capacity for drought prediction using traits and spectra was substantial. In order to maximise trait variation and demonstrate a robust multi-
species approach, we intentionally incorporated a range of crop species including: woody and herbaceous, graminoid and broadleaf, C\textsubscript{3} and C\textsubscript{4}, temperate and arid, and crops grown for their wood, grain, or fruits. Whilst we advocate such an approach for developing an applicable model, we acknowledge that a species- or system-targeted approach could prove even more beneficial for making early predictions of drought stress in the breeding context if multiple cultivars of a single species are under examination.

The techniques we present here will impact crop breeding for water use efficiency and crop management, particularly in water scarce areas. More research will be needed to fully elucidate the utility of our approach, since our multi-species approach was limited to only a small selection of agriculturally relevant species. In future work it will be informative to expand the field system to a wider range of species and indeed a wider range of conditions including additional abiotic and biotic stresses threatening crop production (Claeys & Inzé, 2013). Our approach will be particularly valuable for measurements of biotic stresses, which frequently have no externally measurable indicators, as well as for building upon pre-visual detection of visible stresses such as pathogen damage (Gold \textit{et al.}, 2020). Future research should investigate the applicability of our approach for detecting a broader range of stresses, including combinations of stresses. It will also be important to broaden our technique to a range of genotypes within each species, in order to see if hyperspectral reflectance can detect differences in water stress across crop varieties, to fully realise the breeding potential of our approach. We further envisage the development of our technique in future experiments to encompass canopy- and field-scale spectroscopy utilising existing gantry- and Unoccupied Aerial
System (UAS)- mounted instruments (Smith et al., 2002; Asner & Martin, 2008; Kokaly et al., 2009; Virlet et al., 2017; Coops et al., 2019; Furbank et al., 2019; Gerhards et al., 2019; Shiklomanov et al., 2019; Herrmann et al., 2020; Meacham-Hensold et al., 2020; Yang et al., 2020; Burnett et al., 2021). In future these systems may be used with the novel inclusion of a multi-trait prediction system to quantify complex characteristics such as the drought response at the metabolic level prior to visual detection. Our approach is more effective than one focused on individual metabolites, or a combination of atomic elements (Kunz et al., 2017), due to the intricate nature of the plant metabolic response to drought (Claeys & Inzé, 2013). At the largest scale, successful development and adoption of these predictive techniques using satellite data would contribute additional detail to early warning systems for drought events across the globe (Sutanto et al., 2019), impacting humanitarian relief and development work as well as ecosystem-level risk analyses to protect nature and agriculture against drought.
Supplementary data

Supplementary Figure 1. Example workflow for visual identification of drought.

Supplementary Figure 2. ROC analysis for LDA models.

Supplementary Figure 3. ROC analysis for PLS-DA models.

Supplementary Dataset. Available online at https://doi.org/10.21232/UTK8zaW4.

Data availability

The full raw dataset accompanying this manuscript is available online at EcoSIS (ecosis.org) at https://doi.org/10.21232/UTK8zaW4.

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Author contributions

A.C.B., S.P.S., K.S.E. and A.R. designed the research. A.C.B. led data collection with assistance from K.D. All authors contributed to data analysis. A.C.B. wrote the manuscript with contributions from all authors.
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### Tables

Table 1. Data used to prepare partial least-squares regression (PLSR) models presented in Figure 5.

<table>
<thead>
<tr>
<th>PLSR model</th>
<th>Cal</th>
<th>Val</th>
<th>nComps</th>
<th>Data transformation</th>
<th>Spectra transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>382</td>
<td>97</td>
<td>12</td>
<td>Square-root</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>343</td>
<td>87</td>
<td>13</td>
<td>Square-root</td>
<td>1100-2400nm only</td>
</tr>
<tr>
<td>Protein</td>
<td>397</td>
<td>105</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ABA</td>
<td>67</td>
<td>17</td>
<td>8</td>
<td>Log_{10}</td>
<td>Log_{10}(1/R)</td>
</tr>
<tr>
<td>Amino acids</td>
<td>347</td>
<td>92</td>
<td>10</td>
<td>Square-root</td>
<td>1100-2400nm only</td>
</tr>
<tr>
<td>Proline</td>
<td>348</td>
<td>90</td>
<td>11</td>
<td>Square-root</td>
<td>Log_{10}(1/R)</td>
</tr>
</tbody>
</table>

‘Cal’ and ‘Val’ indicate the number of data points allocated to the calibration (training) and validation (testing) dataset for each PLSR model presented in Fig. 5 (plots in Fig. 5 show the validation data). ‘nComps’ indicates the number of components used in each PLSR model. ‘Data transformation’ indicates transformations performed on biochemical data prior to model building in order to achieve a normal distribution. ‘Spectra transformation’ indicates when spectral wavebands were transformed as log_{10}(1/R) where R is reflectance, in order to improve model fit; this column also indicates that for starch and ABA, wavebands in the restricted range 1100-2400nm, rather than the full range 500-2400nm, were selected to minimise noise.
Table 2. Prediction of plant drought from spectral data using partial least squares discriminant analysis (PLS-DA).

<table>
<thead>
<tr>
<th>Spectral data used in PLS-DA model</th>
<th>Validation accuracy</th>
<th>Kappa value</th>
<th>Number of spectra in training dataset</th>
<th>Number of spectra in testing dataset</th>
<th>Number of components</th>
</tr>
</thead>
<tbody>
<tr>
<td>All glasshouse spectra</td>
<td>66%</td>
<td>0.3224</td>
<td>1316</td>
<td>438</td>
<td>21</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; temporal quartile of glasshouse spectra</td>
<td>59%</td>
<td>0.1847</td>
<td>312</td>
<td>103</td>
<td>13</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; temporal quartile of glasshouse spectra</td>
<td>60%</td>
<td>0.2037</td>
<td>328</td>
<td>108</td>
<td>9</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; temporal quartile of glasshouse spectra</td>
<td>68%</td>
<td>0.3692</td>
<td>394</td>
<td>130</td>
<td>17</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; temporal quartile of glasshouse spectra</td>
<td>94%</td>
<td>0.8716</td>
<td>285</td>
<td>94</td>
<td>21</td>
</tr>
<tr>
<td>All glasshouse data were identified in first column were divided for model training and model testing.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Spectral data used in PLS-DA model</th>
<th>Validation accuracy</th>
<th>Kappa value</th>
<th>Number of spectra in training dataset</th>
<th>Number of spectra in testing dataset</th>
<th>Number of components</th>
</tr>
</thead>
<tbody>
<tr>
<td>All glasshouse data, testing all field spectra</td>
<td>63%</td>
<td>0.2578</td>
<td>1754</td>
<td>321</td>
<td>21</td>
</tr>
<tr>
<td>All glasshouse spectra, testing 1&lt;sup&gt;st&lt;/sup&gt; temporal half of field spectra</td>
<td>71%</td>
<td>0.4199</td>
<td>1754</td>
<td>180</td>
<td>21</td>
</tr>
<tr>
<td>All glasshouse spectra, testing 2&lt;sup&gt;nd&lt;/sup&gt; temporal half of field spectra</td>
<td>53%</td>
<td>0.0455</td>
<td>1754</td>
<td>141</td>
<td>21</td>
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<tr>
<td>All glasshouse spectra, testing first ten days of field spectra</td>
<td>70%</td>
<td>0.4</td>
<td>1754</td>
<td>70</td>
<td>21</td>
</tr>
<tr>
<td>All glasshouse data were used for model training; field data were used for model testing;</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The upper portion of the table shows the results of models trained and tested using glasshouse spectra; the lower portion shows the results of models trained using glasshouse spectra and tested using spectra measured on field-grown *C. pepo*. The percentage accuracy of the model for the validation (testing) dataset, validation kappa accuracy, number of spectra used to train and test the model, and number of model components selected, are presented. AUC values and ROC analyses are published in Supporting Information Fig. S3.
Figure Legends

**Figure 1.** First visible differences between droughted and watered plants. Photographs show droughted and watered plants of each species on the first day when drought could be visually detected (identified in the top row). Soil moisture content is shown for watered (blue) and droughted (red) plants of each species, with standard errors about the mean (n=12 plants per treatment for *C. pepo*; n=8 plants per treatment for *H. annuus, R. sativus, P. canadensis, S. bicolor*). The grey portion of each plot shows the time period before drought could be detected visually. No soil moisture data are available for *C. annuum*.

**Figure 2.** Photosynthesis and stomatal conductance only decrease after drought stress is visible to the naked eye. Panels show net CO₂ exchange per leaf area (a *H. annuus, b R. sativus, c S. bicolor*) and stomatal conductance to water vapour per leaf area (d *H. annuus, e R. sativus, f S. bicolor*). Blue lines denote watered plants; red lines denote droughted and watered plants. Bars show one standard error above and below the mean (n=8 plants per treatment for each species). The grey portion of each plot shows the time period before drought could be detected visually. Black arrows indicate the day on which the first statistically significant difference in A or gₛₘ was detected between droughted and watered plants. A model including all time points revealed highly significant interactions between time and treatment for A and gₛₘ for each species as follows. For *H. annuus*, F = 14.03(1,138), p < 0.001 for A; F = 29.87(1,138), p < 0.001 for gₛₘ. For *R. sativus*, F = 44.32(1,142), p < 0.001 for A; F = 52.41(1,142), p < 0.001 for gₛₘ. For *S. bicolor*, F = 28.02(1,58), p < 0.001 for A; F = 37.31(1,58), p < 0.001 for gₛₘ. Pairwise post-hoc comparisons revealed the following differences. *H. annuus* showed a significant decrease in A and gₛₘ from 26 days into drought, sustained until the end of measurement (on days 26, 28, 29: for A P < 0.05, df 63, 63, 66.4; for gₛₘ P < 0.01, df 55.3, 55.3, 56.2; Fig. 2a,d). *R. sativus* showed a significant sustained decrease in A from day 21 onwards (P < 0.05 on days 21, 23, 28; P < 0.001 on days 30, 33; df 128) and a significant sustained decrease in gₛₘ from day 19 (P < 0.05 on days 19, 21; P < 0.001 on days 23, 28, 30, 33; df 110; Fig. 2b,e). *S. bicolor* showed a significant sustained decrease in A and gₛₘ from day 18 of drought (for A on day 18 P < 0.05, on day 23 P < 0.001, df 48.9; for gₛₘ on day 18 P < 0.05, on day 23 P < 0.001, df 57.1; Fig. 2c,f).

**Figure 3.** The metabolic response to drought varies between species. For key metabolites affected by the drought treatment, percentage changes in droughted relative to watered plants are shown for each species. Red bars indicate an increase in the level of a metabolite under drought treatment; pink bars indicate a decrease under drought treatment. In each case, the x-axis displays days into the drought treatment (with the same axis limits used for all plots) and the y-axis displays percent change (y-axis limits are constant across all species but are adjusted for each metabolite as follows: nitrogen -20 to +220%; starch -100 to +25%; protein -20 to +50%; ABA -100 to +2700%; amino acids -50 to +1100%; proline -50 to +950%). The horizontal black line indicates no change. For each time point, represented by an individual bar, n=12 plants per treatment for *C. annuum* and *C. pepo*; n=8 plants per treatment for *H. annuus, R. sativus, P. canadensis, S. bicolor*. The traits presented here were selected from a suite of biochemical analysis (see Materials and Methods) using a linear model including all species and all biochemical traits, which identified the following overall changes during drought: nitrogen P < 0.05; amino acids P < 0.01; proline P < 0.01; starch P < 0.001; ABA P < 0.001. Protein did not show a significant drought response in the overall model but is included here due to its role as an early metabolic indicator of drought in *C. annuum* and *C. pepo* (Fig. 4).

**Figure 4.** Metabolic signals can indicate drought before it is visible in *C. annuum* and *C. pepo*. Out of six key metabolites, three (starch, protein and ABA) are able to improve drought detection time in *C. annuum* and/or *C. pepo* when compared to visual detection, with starch and ABA signals sustained throughout drought. Metabolites shown are a nitrogen, b starch, c protein, d ABA, e amino acids, f proline. The dashed horizontal line represents no change; bars above the dashed line are faster than visual detection whilst bars below the dashed line are slower than visual detection. Detection time was identified as the first time point with a significant trait difference between droughted and watered plants in each case; n=12 plants per treatment for each species.

**Figure 5.** Partial least-squares regression predicts values of five key metabolites. Model validation plots show the relationship between observed metabolite concentrations (measured using traditional techniques) and predicted
concentrations obtained using partial least-squares regression (PLSR) performed for a nitrogen, b starch, c protein, d ABA, e amino acids, f proline. Circles indicate droughted plants; triangles indicate watered plants; points are coloured according to the species key in panel f. All plots show original, untransformed data. Each model was built using a calibration dataset and tested using a validation dataset; plots show validation data. Details of the datasets are provided in Table 1. Note that for ABA, only data from C. pepo were used for the development of a PLSR model (see Materials and Methods).

Figure 6. Models built using glasshouse data predict leaf metabolite concentrations from spectra in watered and droughted field-grown C. pepo. Partial least-squares regression (PLSR) model coefficients were applied to novel spectra measured on field-grown C. pepo (n=321) and used to predict the following key metabolites: a starch, b protein, c ABA, d amino acids, e proline. Prediction was validated with observed metabolite concentrations for field-grown C. pepo and was successful for starch and protein. Circles indicate droughted plants; triangles indicate watered plants. Models used were the same as those described in Fig. 5, and the same data transformations were performed (Table 1). All plots show untransformed data. Nitrogen data are not available for field-grown C. pepo.

Figure 7. Successful prediction of the class droughted or watered using spectra may be achieved using PLS-DA or a combination of PLSR and LDA. a, best-case scenarios of the speed of drought detection using biochemistry (Fig. 4) and physiology (Fig. 2) relative to detection using the naked eye show that only metabolic traits are sufficient for early detection, but these may be predicted using spectra; b, prediction accuracy of droughted or watered condition using observed biochemical traits in an LDA model and using raw spectra in a PLS-DA model for plants in the main study; c, prediction accuracy of droughted or watered condition using PLSR-predicted biochemical traits in an LDA model and using raw spectra in a PLS-DA model for field-grown C. pepo; d, prediction accuracy of droughted or watered condition using PLSR-predicted biochemical traits in an LDA model and using spectra in a PLS-DA model for field-grown C. pepo during the first ten days of drought.
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![Graphs showing predicted vs observed metabolite concentrations](image)
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**Supplementary Figure 1.** Example method for visual detection of drought stress, displayed here for *P. canadensis*. Photographs of droughted and watered plants were visually compared and the first sign of drought reported – in this case, on day 15 into the drought treatment (yellowing of lower leaves indicated with white ring).

<table>
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<th>Days into treatment</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tbody>
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<td>Droughted plants</td>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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<tr>
<td>Watered plants</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
<td>Days into treatment</td>
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<td>20</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>26</td>
<td>29</td>
<td>34</td>
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<tr>
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<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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</table>
Supplementary Figure 2. ROC-analysis of LDA results for predicting drought from key metabolites, in the main glasshouse experiment. Sensitivity is the true positive rate; 1- specificity is the false positive rate.
Supplementary Figure 3. ROC-analysis of PLS-DA results reported in Table 1 in main manuscript. Results pertain to models built and validated with glasshouse data (a-e) and models built with glasshouse data and validated with field data (f-i). a, main glasshouse experiment, at all times; b, main glasshouse experiment, first temporal quartile; c, main glasshouse experiment, second temporal quartile; d, main glasshouse experiment, third temporal quartile; e, main glasshouse experiment, fourth temporal quartile; f, field validation, at all times; g, field validation, first temporal half; h, field validation, second temporal half; i, field validation, first ten days. AUC values are reported within each plot.