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Development of a diurnal dehydration index for spring barley phenotyping

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Abstract. Spectral and thermal assessments may enable the precise, high-throughput and low-cost characterisation of traits linked to drought tolerance. However, spectral and thermal measurements of the canopy water status are influenced by the crops' soil coverage, the size of the biomass and other properties such as the leaf angle distribution. The aim of this study was to develop a referenced spectral method that would be minimally influenced by potentially perturbing factors for retrieving the water status of differing cultivars. Sixteen spring barley cultivars were grown in field trials under imposed drought stress, natural drought stress and irrigated conditions. The relative leaf water content of barley plants declines diurnally from pre-dawn until the afternoon, and other plant traits such as the biomass change little throughout the day. As an indicator of the current drought stress, pre-dawn and afternoon values of the relative leaf water content were assessed spectrally. Diurnal changes in reflectance are only slightly influenced by other perturbing factors. A new spectral index (diurnal dehydration index) was developed by using the wavelengths 730 and 457 nm collected from an active spectrometer. This index allowed the differentiation of the drought tolerance of barley plants. The diurnal dehydration index was significantly related to final biomass, grain yield and harvest index and significantly different between cultivars. Compared with other indices, the diurnal dehydration index offered a higher stability in retrieving the water status of barley plants. Due to its diurnal assessment, the index was barely influenced by the differences in cultivars biomass at the time of measurement. It may represent a valuable tool for assessing the water status or drought tolerance in breeding nurseries.

Additional keywords: abiotic stress, drought tolerance, phenomics, high throughput, precision phenotyping, spectroscopy.

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Introduction

Water status can be defined as the mass and potential energy of water that is currently stored in a canopy, a single plant or in the tissue of a plant organ. Changes in water status occur if the flux of water from the soil does not coincide with the water losses from the plant due to transpiration. Because this is frequently the case under natural conditions, water status in the plant is dynamic. Fluctuations in water status exist in a diurnal course (Van Iersel and Oosterhuis 1995; Schmidhalter et al. 1998), as well as in drying soils due to lack of precipitation.

Water status measurements should offer precise information about the drought and osmotic stresses currently affecting a plant. Leaf relative water content (RWC) and leaf water potential varied inversely with diurnal trends of air vapour pressure deficit, air temperature, solar radiation and net radiation in barley (Hordeum vulgare L.). The plants were grown in soil near field capacity and near the permanent wilting point on a cool, mostly cloudy day and a warm, mostly clear day (Millar et al. 1971). Diurnal changes in the leaf water potential and the RWC in response to the diurnal evaporative demand were also observed by Girma and Krieg (1992). They measured expanding and the uppermost fully expanded leaves before flowering and fully expanded leaves during the grain filling period of sorghum (Sorghum bicolor L. Moench) plants grown in the field under variable water supplies.

Various methods to detect drought stress by water status measurements have been adopted for physiological investigations and to guide irrigation management. Water content, which is defined as the leaf water mass (FW minus DW) divided by FW, is easily measured but difficult to interpret physiologically (Weber and Ustin 1991). The method is sensitive to differences in DW at the time of sampling and to loss of water before the initial FW is taken. Physiologists prefer measurements of the RWC, defined as the leaf water mass at harvest (FW – DW) divided by the leaf water mass at full hydration (turgescent weight – DW) (Weatherley 1950). In a typical diurnal cycle, the highest RWC occurs before sunrise, and the lowest occurs in early to mid-afternoon (Weber and Ustin 1991). Advantages of using RWCs are that changes in DW during the day are removed and that physiological comparisons are more interpretable.
Because classical and destructive water status measurements are tedious and time-consuming, as well as impractical for screening large numbers of cultivars due to the diurnal changes, non-invasive approaches could offer an alternative to trace the diurnal changes. Among such methods, dendrometers measure the diameter of plant organs such as stem or leaf to indicate the water status (Zimmermann et al. 2008). The diameter of a plant organ changes during the day due to changes in cell turgor. The diurnal course of leaf angle in tomato plants tracked by digital picture analyses has shown to be related to water status (Font and Körösi 2005). However, these methods are restricted to a few plants and will not allow screening a large number of cereal lines grown under field conditions.

Non-destructive remote sensing offers a viable alternative for screening many cultivars in a short time, thus potentially enable high-throughput phenotyping. The method presented here is based on the use of spectral analyses and the impact of tissue hydration on spectral reflectance. Carter (1991) measured the reflectance of a dehydrating Magnolia grandiflora L. leaf. The reflectance of the leaf increased considerably over the measured visible and infrared spectrum of 400 to 2500 nm while drying from a RWC of 100 to 5%. The most pronounced changes in reflectance occurred in the water absorption bands at 1200, 1450 and 1930 nm. The index \( R_{1300}/R_{1450} \) was linearly related to the RWC. The water index \( R_{1300}/R_{1450} \) was also successfully used by Seelig et al. (2008) to measure RWCs in a living bean leaf between 100 and 65% and in a desiccated leaf under 65% RWC. Reflectance was measured repeatedly in detached cotton leaves desiccating from a RWC of 99–80% (Bowman 1989). Infrared reflectance increased, although visible reflectance change relatively little. The closest relationships between relative leaf water content and reflectance were found at 810 nm (\( r^2 = 0.79 \)), while they were weaker for 1665 nm (\( r^2 = 0.74 \)) and 2212 nm (\( r^2 = 0.65 \)). Other authors (Ceccato et al. 2001) preferred the simple ratio index \( R_{1600}/R_{820} \) nm for assessing the leaf equivalent water thickness expressed in quantity of water per unit area (g cm\(^{-2} \)) in leaves of the species Acer pseudoplatanus L., Artenicia vulgaris Lam., Morus alba L. and Prunus laurocerasus L.

In a breeding setting, most often, whole plant canopies in plots rather than single leaves are measured spectrally. Significant correlations of a water index (WI) (\( R_{9600}/R_{970} \)) to canopy temperature depression (\( r^2 = 0.64 \)), biomass at harvest (\( r^2 = 0.68 \)) and grain yield (\( r^2 = 0.73 \)) were found in a field trial with six wheat genotypes grown under well-watered conditions in northern Mexico (Gutiérrez-Rodriguez et al. 2004). Significant correlations of a normalised water index (NWI) (\( R_{970} = R_{980} + R_{980} \)) to the leaf water potential were obtained at single growth stages and across growth stages (Gutiérrez et al. 2010). However, relationships between the NWI and the RWCs were not significant at single growth stages but only across growth stages. The plant water content of winter wheat (Triticum aestivum L.) could be estimated by using the red edge band information (680–780 nm) and the leaf water potential could be estimated by using a WI and a NWI including the waveband at 970 nm (\( r^2 = 0.34–0.75 \)) (Liu et al. 2004). The normalised spectral indices using the wavelengths 490, 510 and 780 nm were closely related to the canopy water content of different wheat cultivars grown in a two-factorial field trial with different watering regimes (Mistele et al. 2012). spectral indices were highly correlated with the canopy water mass in a set of tropical maize hybrids and allowed to differentiate drought stress levels (Winterhalter et al. 2011). The reflectance ratio \( R_{750}/R_{720} \), the normalised difference vegetation index and the reflectance ratio \( R_{780}/R_{550} \) spectral index were significantly correlated with the FW, the water content of the aboveground biomass and the water potential of the youngest fully developed leaf of wheat plants (Hackl et al. 2013). Based on a sensitivity analysis of a radiation transfer model the shortwave infrared (SWIR)-range was found sensitive to equivalent leaf water thickness independent of the species in satellite-based remote sensing of vegetation (Ceccato et al. 2002b).

Short-term exposure of dense stands of plants to increasing or decreasing artificial light intensities in a growth chamber was used to simulate diurnal changes and allowed to identify significant relationships (\( r^2 \)-values of 0.74–0.92) between the leaf water potential and spectral indices ((\( R_{940}/R_{960} \)/NDVI; \( R_{940}/R_{960} \)) for wheat and maize plants (Elayed et al. 2011). The exact relationships found, however, were influenced strongly by the date of measurement or water stress induced. Although spectrometric measurements have the potential for fast and non-destructive measurement of the plant water status, they still have drawbacks. Spectral characteristics of plants and plant stands change with increasing biomass, and therefore vary in growing canopies across plant development stages. Environmental factors like temperature, radiation and air humidity shift water status parameters like leaf water potential and RWC thus impacting calibrations. A problem in passive spectroscopy is its dependency on solar angle. In contrast, active spectrometers are reported to be independent of light conditions (Kipp et al. 2014b) and can also be used at night.

In summary it is difficult to establish global spectral relationships in measuring the RWC which are applicable to various development stages and across different environments. It remains unclear whether changes in the water status can reliably be detected spectrometrically over time and whether such measurements primarily reflect changes in the aerial plant biomass (Elayed et al. 2011). If no reliable absolute measurements of water status can be made without individual calibration, it might still be possible to use relative differences in the spectral properties of genotypes to detect differences in their water status.

A central problem in water status estimation of canopies by non-imaging spectral analyses is that neither spectra nor derived indices react to the water status alone. Canopy spectral variability is frequently influenced by several soil and canopy traits such as soil cover, soil background reflectance, leaf area index, leaf thickness, biomass, leaf angle distribution, chlorophyll and pigment content and water content. This explains the sometimes poor performance when used to detect a single trait and is of particular relevance when trying to transfer spectral indices to other sites, plants or cultivars. Under field conditions, all mentioned traits change in the course of plant growth. Thus, the background for measuring a trait of interest is not constant across repeated-measurements during the vegetation period. However, the fact that the water status changes during a single day while other growth parameters change instead over the course of days, weeks and months can be exploited.
The specific research goals of this work were to (i) systematically retrieve reflectance indices in the range from 380 to 1000 nm that discernibly react to the water status of spring barley, rather than applying single known indices; (ii) stabilise and improve such water indices by using the diurnal course of the water status by referencing daytime spectral measurements with pre-dawn measurements, reducing the problem of confounding effects of other cultivar traits such as biomass when measuring many cultivars simultaneously; (iii) evaluate the feasibility of the selected index for the high-throughput detection of relative differences of drought tolerance of genotypes in a breeding nursery, and (iv) relate the measured drought tolerance characteristics to important agronomic traits such as grain yield.

**Materials and methods**

**Rain-out shelter experiments**

In the seasons 2010, 2011 and 2012, spring barley cultivars were grown using an automatic rain-out shelter to impose artificial drought. The investigation site is located at the Dürnast research station, belonging to the Technische Universität München, Germany. Barley plants were grown under open sky and natural weather conditions. Only in the case of rain, the rain-out shelter closed automatically and kept the soil and plants dry. The soil under the rain-out shelter is characterised as calcaric cambisol consisting of silty loam. On a volumetric basis, the field capacity is at 42% (v/v) with the permanent wilting point at 20% (v/v), resulting in a plant-available field capacity of 220 mm m$^{-1}$ soil depth. The rooting depth for fully developed spring barley was assumed to be at 1.3 m, based on previous measurements of water extraction in the subsoil. The plant-available field capacity in the rooting depth thus amounts to 286 mm.

The rain-out shelter trial was arranged in a randomised split-block design with the two factors water supply and genotype. Water supply was applied by sprinkler irrigation with spray nozzles mounted with 50 cm distance on a moving bar at 1 m above the soil surface. In 2010 and 2011, irrigation was applied to half of the trial plots with irrigation triggered at a soil matric potential of −700 hPa in 30 cm soil depth. The irrigated part of the field trial served as the control treatment. The remaining half was irrigated only once for emergence and then kept dry during the rest of the season. The plants in this drought-stressed treatment had to rely entirely on soil water reserves and were therefore subjected to increasing drought stress during the season. At the end of the season in 2010, the soil matric potential reached −1500 hPa at a depth of 30 cm and a soil matric potential of −1250 hPa averaged over the whole rooting depth. The individual plots consisted of eight rows spaced at 15 cm and were 1.8 m long. The sowing density was 330 kernels m$^{-2}$.

In 2012, the whole area under the rain-out shelter was used to induce drought stress, while the control was grown rain fed outside the rain-out shelter to maximise space for the drought stress experiment. Because 2012 was a season with sufficient rainfall, no supplemental irrigation had to be applied to the outside control treatment.

The investigated genotypes consisted of a set of 16 German cultivars, the eldest being released in 1895 and the youngest in 2007. This set was chosen to represent the breeding progress in spring barley over the last 120 years, as well as to provide a large genetic variability in architecture, yield, water demand and drought tolerance. The cultivars were grown in at least four repetitions, for 16 cultivars × four replications × two treatments, totalling 128 plots.

In addition to the rain-out shelter experiments, rain fed field trials were established in 2011. Trials were arranged in a split-block design with the same 16 cultivars in three repetitions. The total number of plots was 48. The plots consisted of 14 rows and had a length of 10 m each. Agronomic traits such as grain yield, straw yield, biomass, harvest index, thousand grain weight and grain number/ear were determined. In 2011, natural drought occurred during the vegetative phase. From 5 April 2011 to 25 May 2011, only four significant rainfall events (>3 mm) occurred.

In the rain-out shelter and field trial experiments, residual mineral soil nitrogen levels were measured before sowing. Based on the residual nitrogen levels, additional mineral fertiliser was applied up to a level of 130 kg N ha$^{-1}$, in one application before sowing. All other nutrients were available in adequate amounts in the soil. Herbicide and fungicide treatments were applied when necessary. To reduce the lodging risk, especially of the old tall cultivars, the growth regulator ‘Trinexapac’ (Syngenta AG, Basel, Switzerland) was applied at the initiation of shooting (BBCH 31).

**Water status measurements**

Destructive reference measurements of the water status of the plants in the plots were made repeatedly during each season. RWC measurements were carried out on warm, sunny days during the warmest hours, from 1300 to 1600 hours. The youngest fully developed leaves were used for the measurements following standard procedures (Smart and Bingham 1974). Immediately after cutting, the leaf fresh weight was determined on-site by means of a fine balance. Then, the cut leaves were placed in stoppered tubes with their bases immersed in water and were kept there until full turgidity was reached, at which time they were re-weighed. Finally, the DW was determined after oven-drying at 60°C. RWC was calculated as follows:

$$\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100. \quad (1)$$

To test an alternative method, leaf water content (LWC) was determined instead of the RWC. The LWC was calculated as follows:

$$\text{LWC} = \frac{\text{FW} - \text{DW}}{\text{FW}} \times 100, \quad (2)$$

where TW is turgid weight.

To measure the leaf water potential, a pressure chamber (PMS Instruments, Corvallis, OR, USA) was used. Pressure was read within 1 min of leaf removal from the plant and the leaf water potential was determined from the average value obtained from three fully expanded leaves of similar age.

Canopy surface temperature was determined in the field trials with two Heitronics KT15D infrared thermometers (Heitronics GmbH, Wiesbaden, Germany). The thermal
The spring barley crop was harvested in the rain fed test at the Agronomic parameters. Measurements were averaged for the larger plot size.

Three-dimensional compounds were mounted on a carrier vehicle, PhenoTrac 4 from the Chair of Plant Nutrition, TUM, viewing the canopy from two opposed oblique views at an angle of 45° from nadir. Canopy temperature was assessed by averaging the measurements from both devices. The oblique view increases the biomass fraction and decreases the soil fraction in the field of view. Measuring from opposite sides allows the inclusion of information from sun-lit and shaded canopy.

Spectral measurements were obtained using an active, hyperspectral sensor (customised device, tec 5, Oberursel, Germany), which collects information at 91 wavebands ranging from 380 to 993 nm and with a spectral resolution of 6.6 nm and an optical aperture of 11.4° (Erdle et al. 2011). The light source of the active sensor was a xenon flash lamp. The device was initially calibrated using a white PTFE-surface in a distance of 1 m. Reflectance was calculated by dividing the incoming light intensity by the light intensity reflected from the reference surface. The sensor was fixed in a nadir orientation at 1.5 m above ground on the carrier vehicle. Measurements were taken twice a day, once at night, chiefly pre-dawn, and once in the early afternoon, at the hottest time of the day. The active spectrometer used a pulse-modulated system that is independent from and not affected by ambient light. Measurements of reflectance were taken at ambient light shortly before and simultaneously with the active light source was retrieved. The night measurements were averaged, whereas 50–65 measurements were averaged for the larger field trial plots.

### Agronomic parameters

The spring barley crop was harvested in the rain fed field trial on 20 July 2011. The trials were harvested with a plot combine harvester. Total grain yield was weighed, samples were oven-dried to determine grain water content on a gravimetric basis and yield was expressed as dt ha⁻¹, normalised to a water content of 14% (w/w). Straw was picked up by a forage wagon, dried and weighed. Straw yield was expressed as dt ha⁻¹ dry matter. Biomass yields represent the sum of grain and straw yields. Harvest index (HI) was calculated as ratio of grain yield divided by biomass. From each plot’s biomass a sample of 100 spike-bearing culms was counted and weighed. The number of culms per m² was calculated by the formula:

$$C = \frac{100 \times SW}{0.96 \times W},$$  

where C is the number of culms m⁻², SW is sample weight and W is total weight, the constant 0.96 represents an adjustment factor for the plot size.

Thousand grain weight (TGW) was determined using a seed counter and a balance. Grain number per ear was determined as biomass × TGW × HI/number of tillers.

On 28 June 2012, biomass sampling was performed in medium milk-ripe (growth stage BBCH 75 according to the Federal Biological Research Center for Agriculture and Forestry 2001), drought-stressed spring barley in the rain-out shelter trial. The result was expressed as dt ha⁻¹ dry matter.

### Statistical analyses

Regression and correlation analyses as well as analysis of variance and t-tests were conducted, using the software R (R Foundation, Vienna, Austria). The least significant difference test was applied using the software SPSS ver. 20.0 (IBM, Armonk, NY). Contour diagrams showing the coefficient of determination (r²) for the relationships between the investigated parameters such as RWC and the narrow band normalised differential spectral indices were calculated from all possible two-band combinations in the range of 380–993 nm using R and visualised using the lattice package for R.

### Statistical analyses: calculation of the diurnal dehydration index

In the years 2011 and 2012, spectral reflectance was measured in all plots in the rain-out shelter trial as well as in the field trial at pre-dawn and in the afternoon. Relative water levels were assessed simultaneously with spectral measurements in the rain-out shelter trial in the afternoon. Turgid weight measurements of re-hydrated leaves were assumed as being equivalent to pre-dawn measurements. At night mildly drought stressed plants equilibrate with wetter patches in the soil, and usually reach RWCs close to 100% (Schmidhalter et al. 1998). Therefore it could be resigned from measuring RWCs at pre-dawn.

Spectral reflectance curves measured from all plots at afternoon were used to calculate binary normalised differential spectral indices of the type (R₁ − R₂)/(R₁ + R₂) for all possible combinations of the 91 single wavebands. The same was done with spectral reflectance curves measured at pre-dawn. To reduce the impact of morphological differences among cultivars, the afternoon spectral measurements were normalised to the pre-dawn measurements. This was done by dividing the normalised differential spectral indices from the afternoon measurement by the corresponding indices from pre-dawn. Thus a new index was developed, the diurnal dehydration index (DDI), which is given in Eqn 4:

$$\text{DDI} = \frac{((R_{1\text{night}} - R_{2\text{night}})/(R_{1\text{night}} + R_{2\text{night}}))}{((R_{1\text{day}} - R_{2\text{day}})/(R_{1\text{day}} + R_{2\text{day}}))},$$  

where R₁ is the wavelength reacting sensitive to the water status and R₂ is the reference wavelength used to stabilise the index.

### Statistical analyses: analysis of variance of the effects of canopy temperature and cultivars on the diurnal dehydration index

An analysis of variance was conducted including the factors cultivar, DDI and canopy temperature (see Table 1). This test was to determine whether the DDI could be applied for
Table 1. Analysis of variance of the effect of canopy temperature and cultivar on the diurnal dehydration index

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F-value</th>
<th>P (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canopy temperature</td>
<td>1</td>
<td>0.1181</td>
<td>29.551</td>
<td>5.492e-05</td>
<td>**</td>
</tr>
<tr>
<td>Cultivar</td>
<td>15</td>
<td>0.4916</td>
<td>0.03277</td>
<td>8.198</td>
<td>6.810e-06</td>
</tr>
<tr>
<td>Canopy temperature × cultivar</td>
<td>15</td>
<td>0.0714</td>
<td>0.00476</td>
<td>1.19</td>
<td>0.3661</td>
</tr>
<tr>
<td>Residuals</td>
<td>16</td>
<td>0.064</td>
<td>0.0031</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Significance differences are indicated: ***, P < 0.001; **, P < 0.01; *, P < 0.05; n.s., not significant (P > 0.05).

Table 2. Relative Confidence Intervals (CI%) of drought stress traits measured on May 26, 2011 in an irrigated trial and on June 28, 2011 in a rain fed field trial

<table>
<thead>
<tr>
<th>Trait</th>
<th>CI% irrigated</th>
<th>CI% rain fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative water content</td>
<td>3.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Normalised index</td>
<td>5.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Diurnal drought index</td>
<td>10.1</td>
<td>6.5</td>
</tr>
<tr>
<td>Canopy temperature</td>
<td>–</td>
<td>2.3</td>
</tr>
</tbody>
</table>

assessing water status independent of the cultivar. Data from the rain fed field trial on 28 June 2011, a day with a maximum air temperature of 27.3°C, were used. Canopy temperature was used here as an alternative, indirect indicator of the water status.

**Statistical analyses: calculation of confidence intervals for the measurements of RWC, the diurnal dehydration index and a normalised index**

To compare the measurement accuracy of the used water status indicators, confidence intervals of the RWC, a normalised differential spectral index (NDSI), based on reflectance at 457 and 730 nm and using day data only, and the DDI were calculated for the rain-out shelter trial on 26 May 2011. For comparison, confidence intervals of the canopy temperature (T), the normalised index and the DDI were calculated for the rain fed field trial on 28 June 2011. Calculation of confidence intervals was done as indicated by Eqn 5:

\[
CI\% = \frac{\sigma_x * 100}{\sqrt{n} * x}, \quad (5)
\]

where \(\sigma_x\) represents the standard deviation of the measurements of RWC, IX, DDI or T. The number of replications per cultivar was \(n = 4\) in the irrigated rainout shelter trial and \(n = 3\) in the rain fed field trial and \(t = 3.18\) for \(n = 4\) and \(t = 4.3\) for \(n = 3\), where \(t\) indicates the quantile of Student’s distribution.

The standard deviations used in the calculation were derived from the residuals of linear fits of all single measurements in each field trial by using 31 plots in the irrigated trial and 48 plots in the rain fed trial, and they represent the quality of the measurement. The number of replications \(n\) used in Eqn 4 was based on the number of replications per cultivar, so the confidence intervals represent the accuracy of water status measurements for a single cultivar. Confidence intervals were normalised to the average measurements (Eqn 5, second term) and are expressed as percentages of the average in Table 2.

**Results**

**Diurnal changes in reflectance measurements of the water status in single plots**

The reflectance curves shown in Figs 1 and 2 show a pronounced increase of reflectance from visible to near infrared light. The generally higher reflectance in the near infrared is caused by structural properties of the canopy causing a higher backscattering of the near-infrared wavebands. The reflectance curves indicate a generally lower reflectance in the visible wavebands (400–690 nm, with a small peak observed in the green waveband region) caused by light absorption of pigments.

Comparing the reflectance curves for the irrigated (Fig. 1) and drought-stressed treatment (Fig. 2) shows that the reflectance in the near infra-red (NIR) was higher in the irrigated treatment, at ~68% compared with 55% in the stressed treatment.

In both treatments, the reflectance curves shifted during daytime. The reflectance curves started at a low level at dawn, increased until ~1500 hours, and decreased again towards the evening. Whereas small changes occurred in the visible range, more pronounced changes in reflectance were observed in the NIR range. The shifts in reflectance can be traced back to changes in water status, at 0630 hours the leaf water potential was ~0.73 MPa in the well watered canopy and ~0.83 MPa in the drought-stressed canopy. At 1500 hours a RWC of 75% and a leaf water potential of ~1.43 MPa was reached in the irrigated treatment, and a RWC of 72% and a leaf water potential of ~1.64 MPa was reached in the stressed treatment. With the more pronounced dehydration occurring in the stressed treatment, the reflectance shifted more markedly in this treatment.

Correlations between leaf water potential and the normalised index \((R_730 - R_{457})/(R_730 + R_{457})\) resulted in coefficients of determination of \(r^2 = 0.74\) in case of the irrigated treatment and \(r^2 = 0.70\) in case of the drought stressed treatment. A major part of the diurnal spectral variability can be explained by changes in water status.

**Pre-dawn and afternoon spectral assessments of the water status in the rainout shelter and field experiments**

A contour map (Fig. 3a) shows the correlations of all possible binary indices from hyperspectral measurements done at daytime only with RWC. The wavelengths \(R_1\) and \(R_2\) are represented on the x- and y-axis respectively. Two distinct areas show higher coefficients of determination compared with its surroundings; one area could be identified in the visible range combining wavelengths from 530 to 605 nm (green and yellow) with wavelengths of ~460 nm (blue) and another one in the near infrared range combining wavelengths from 700 to 930 nm (NIR) with wavelengths of ~460 nm (blue). Combinations of the wavelength 967 (water absorption band) with most of the measured wavelengths also show correlations with RWC.

Accordingly all possible combinations of the DDI (see Eqn 3) were calculated, and correlated with the RWC. The DDI represents the relative shift of normalised differential spectral indices due to dehydration. The resulting coefficients of determination \((r^2)\) were visualised in contour maps depicting optimised wavelength combinations (Figs 3b, 4a).
Fig. 4b shows a combined contour map containing the average coefficients of determination of four contour maps derived from daytime and night-time measurements. Similarly like in the contour map with data from daytime only, two distinct areas show higher coefficients of determination compared with its surroundings; one area could be identified in the visible range combining wavelengths from 530 nm to 570 nm (green) with wavelengths of ~450 nm (blue) and another one in the near infrared range combining wavelengths from 710 to 930 nm (NIR) with wavelengths of ~450 nm (blue).

From all possible index combinations shown in the contour maps from single measurements, the best index with the highest coefficient of determination was selected (Table 3). Optimised indices presented in Table 3 shifted in location and quality (R^2-values) from measurement to measurement. For the measurements using night and day data, optimised indices differed from the measurements using day data only. For example using day data from 28 June 2012, the WI that combines the band at 967 nm, where light is absorbed by water, with that at 934 nm was selected as optimised index. This index is similar to a WI described by Peñuelas et al. (1997), which uses wavelengths at 970 and 900 nm. However, in other measurements, the combinations of NIR and blue, or green and blue wavebands turned out to be the best. To retrieve a more stable index leading to significant results for the majority of the measurements, the following steps were performed: data from the contour map derived from measurements of the drought-stressed treatment on 26 May 2011 and two contour maps from 28 June 2012 (data not shown) were used for calculating a combined average matrix. The index obtained from the 730 and 457 nm waveband regions turned out to have the highest average coefficient of determination in this matrix (r^2 = 0.25). This DDI was applied at two single dates included in the averaging (results in Table 3). For the measurements taken on 28 June in the drought-stressed treatment, the index showed a correlation with RWC, with a coefficient of determination of r^2 = 0.50. A significant linear regression obtained from this dataset is shown in Fig. 5. The regression line shows a negative slope, indicating that larger shifts in the chosen index between night and day coincide with lower relative water levels and thus increased tissue dehydration. The regression function relates the DDI to the RWC. Applying this index and function to other measurements under conditions of mild drought stress will mostly not lead to absolute values of RWC. However it can indicate relative differences in the current water status of the measured plots.

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**Fig. 1.** Hyperspectral reflectance measurements of an irrigated spring barley plot at anthesis (cultivar: Apex) at different times of day obtained on June 11, 2010, a cloudless and warm day (T_{max}: 26.1°C).

**Fig. 2.** Hyperspectral reflectance measurements of a drought-stressed spring barley plot at anthesis (cultivar: Apex) at different times of day obtained on June 11, 2010, a cloudless and warm day (T_{max}: 26.1°C).

**Fig. 3.** (a) Contour plots depicting the relationship between coefficients of determination of normalised spectral indices (x-axis, R1; y-axis, R2) measured at daytime with relative water content in an irrigated field trial on 26 May 2011; (b) coefficients of determination of normalised spectral indices measured at night and daytime with relative water content in an irrigated field trial on 26 May 2011.
Fig. 6a shows a contour map containing coefficients of determination of above ground dry weight with normalised spectral indices measured at daytime on 31 May 2011 in a drought-stressed field trial on 28 June 2012. (b) Average coefficients of determination of normalised spectral indices measured at night and during daytime with relative water content from irrigated and drought-stressed field trials measured on 26 May 2011, and 28 June 2012.

Table 3. Referenced optical measurements conducted in field trials: R1, R2, wavelength of normalised spectral indices of the type (R1 – R2)/(R1 + R2) or ((R1\text{day} – R2\text{day})/(R1\text{day} + R2\text{day})) ((R1\text{night} – R2\text{night})/(R1\text{night} + R2\text{night}))

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Night and day measurements

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Calibration night and day measurements

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Fig. 6a shows a contour map containing coefficients of determination of correlations of above ground dry weight with normalised spectral indices measured at daytime on 31 May 2011 in a drought-stressed field trial. Both NIR/visible light (VIS) and VIS/VIS indices responded highly sensitive to biomass. Diurnal indices in Fig. 6b show only very few wavelength combinations that are impacted by biomass. There was no significant correlation between above ground DW and the reference measurement of RWC, but a weak significant correlation was obtained between above ground DW and temperature ($r^2 = 0.16$) at the same day. However, this correlation is probably indirect and can be traced back to enhanced growth of plants with better access to water and higher stomatal conductance resulting in lower surface temperatures.

Analysis of variance of the effects of canopy temperature and cultivars on the Diurnal Dehydration Index

The analyses of variance indicated a significant influence of the canopy temperature and the cultivars on the DDI. No interactions between canopy temperature and cultivars were found.

Confidence intervals of water status indicators

The relative confidence intervals from two field trials for the water status indicators RWC, a normalised index (IX), based on reflectance at 457 and 730 nm and using day data only, and the DDI are shown in Table 2. The RWC and canopy temperature showed the smallest standard deviations and relative confidence intervals in both trials, whereas the normalised index and particularly the DDI showed increased confidence intervals.
Least significant differences of the DDI for differentiating cultivars

An l.s.d. test was applied on the DDI data measured for all 16 cultivars in the rain fed field trial. Measurements were conducted at night and day on 28 June 2011. The maximum temperature of this day was 27.3°C. It followed a drought period in late April and May that was interrupted by some rainfalls in June. The combination of warm weather and dry soil caused mild drought-stress. Table 4 compares the cultivars with each other and indicates significant differences of the DDI.

The coefficient of determination obtained from the correlation between the DDI and the year of registration of the cultivar (Table 4) was $r^2 = 0.38$

Relation of drought stress tolerance to yield and other agronomic parameters

Table 5 shows the coefficients of determination of correlations of the DDI measured on 28 June 2011 (BBCH 85) with different agronomic traits in the rain fed field trial that was subject to natural drought during the vegetative phase. Significant linear relationships between the DDI and grain yield were found in the rain fed field trial that was subjected to natural drought stress during the late vegetative and early generative phase (Table 5). In the rain-out shelter trials, no significant correlations were obtained.

Discussion

Assessment of the DDI

The physiological basis of the DDI should be understood and the technical feasibility of the optimum DDI must be assessed for its use in cultivar trials. Its properties therefore need to be compared with indices that are based on day data only and with the traditional destructive methods for measuring the water status. The environmental conditions of a successful application of the DDI are discussed below.

Physiological basis of the DDI

The reflectance measurements presented in Figs 1 and 2 were taken throughout a diurnal course in single plots. Generally higher reflectance in the NIR range could be found in the irrigated treatment (Fig. 1) compared with the drought-stressed treatment (Fig. 2). This reflects the higher biomass found for the irrigated crop compared with the drought-stressed crop. The cumulative effect of the previously imposed drought stress led to a pronounced decrease in biomass. The irrigated plot had 1202 ear bearing culms at harvest, whereas the stressed plot had 745 ear bearing culms.

Reflectance, especially in the NIR-range, started at a low level at pre-dawn and increased until the afternoon. A technical cause of these shifts can be excluded because reflectance was measured by an active device. This device is not affected by the zenith angle of sunlight and is very little affected by shifts in temperature (Kipp et al. 2014a). The shift is, therefore, caused by the dehydration of the plants during the warm, dry hours and by rehydration towards the evening. At pre-dawn, cells are hydrated, and apoplastic spaces are partly filled with water; the water in the tissue is under little tension. As the tissue dehydrates...
Table 4. Arithmetic means of the diurnal dehydration index \( (n = 3) \) and significant differences between cultivars according to a l.s.d.-test

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Abbreviations: Pe, Perun; Ur, Ursa; Bar, Barke; Wi, Wiebke; Eu, Eunova; Tr, Trumpf; St, Streif; Si, Sissy; Vi, Victoriana; Ap, Apex; Pf, Pflugs In.; Be, Beatrix; Dj, Djamila; Is, Isaria; H. F, H. Franken; Bav, Bavaria. NR, not registered. Significant differences are indicated: *, \( \alpha = 0.05 \)

Table 5. Relationships between the diurnal dehydration index (DDI, measured on 28 June 2011) and different agronomic traits (measured at harvest) are indicated by coefficients of determination

Significant differences are indicated: ***, \( P < 0.001 \); **, \( P < 0.01 \); *, \( P < 0.05 \); n.s., not significant (\( P > 0.05 \))

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during the day, water in the intercellular spaces and, due to osmotic adjustment, to a lesser degree in the cells, starts to retreat and forms menisci of larger surface (Elsayed et al. 2011). The increasing surface of the water/air boundary layer might cause the increased scattering and reflectance especially of near-infrared radiation and to a lesser extent of visible radiation. Because less green light is absorbed by pigments than red or blue light, its path length through the canopy is longer and more scattering at boundary layers is to be expected.

In the stressed treatment (Fig. 2), dehydration was more pronounced than in the irrigated treatment. Accordingly, reflectance also shifted more in this treatment. A greater shift in reflectance between night and day is therefore an indicator of drought stress.

The indices shown in the contour maps, including only visible wavelengths. Information found at the red edge wavelength 730 and the blue wavelength 457 nm was chosen as the optimised DDI, indicating the closest relationship to the RWC (Table 3). Additional indices including neighbouring wavelengths also correlate with the RWC (Fig. 4b).

Repeatability and significant relationships with RWC (Table 3) – a well established indicator of water status and drought stress (Weatherley 1950; Smart and Bingham 1974) – show, that the DDI is a valid proxy of water status. The measurement of the DDI in field trial plots was feasible and could replace tedious destructive measurements of the RWC. The DDI can therefore be used for retrieving relative differences in relative leaf water contents. Absolute values can be achieved only in calibrated experiments, due to the changing conditions in repeated field measurements. For crop phenotyping, however, a relative comparison of genotypes is sufficient, not requiring absolute values of RWCs.

Comparison of the Diurnal Dehydration Index

with indices based on daytime data

Relationships of spectra with the RWC can also be obtained using daytime data only (Fig. 3a). However, information related to the water status is more clearly found in the contour maps when night data are included (Figs 3b, 4a). Therefore, most of the tested indices showed a better performance indicated by higher coefficients of determination when night data were included.

A partial explanation for this improvement is reflected in information found in Fig. 6, where biomass measured as above ground dry mass was used as the reference instead of the RWC. Using daytime data only (see Fig. 6a), differences in biomass can clearly be retrieved from normalised spectral indices. Coefficients of determination were generally higher than for water status assessments. The spectral zones that contain information on biomass were partly found at the same wavelengths that contain information about water status.

The contour map that combines the results from day and night measurements (Fig. 6b) contains almost no relevant information for retrieving biomass. Although some biomass related
information is still contained and an influence of biomass on the retrieval of the DDI cannot fully be excluded, such an impact is largely reduced compared with indices using daytime data only. When using daytime spectral information only, information about the water status is partly masked by the predominant influence of biomass on reflectance. Therefore, combining daytime measurements with night-time measurements is advantageous. With no or little change in total biomass during a single day, shifts in reflectance can mostly be ascribed to changes in water status.

Suitable phenological phases and timing of optical water status retrieval

Significant relationships between optical measurements of the DDI and RWC could be established in the phenological stages of heading (BBCH 56) and the onset of maturity (BBCH 85) (see Table 3). Significant correlations of the DDI with canopy surface temperatures, measured by the thermal infrared sensor, could be established at the onset of maturity (BBCH 85). However, in earlier phenological stages such as the tillering phase, no relationships between reflectance and the water status or canopy temperature could be obtained. This may be related to the intensity of stress and/or the need to have an established crop stand. There is a smaller chance to apply the DDI in early growth stages before shooting. In contrast, in late plant-growth stages such as the maturation stage, the water status will be dominated by the degree of senescence, which is superimposed on any diurnal change. Optical retrieval of water status is probably best conducted between BBCH 54 and 87.

For selecting drought-tolerant genotypes, measurements of the water status are particularly useful at anthesis and early grain filling (BBCH 61 to 83), when cereal crops react to drought stress with particular sensitivity. With regard to daytime, spectral reflectance measurements are best conducted at the most severe stress situation experienced by the plant, which normally coincides with measurements obtained between midday and mid-afternoon. In the afternoon, conditions of global irradiance and heat are mostly more stable than before noon, and measurements are least affected by diurnal changes.

Independency of the water status measurements from miscellaneous other cultivar traits

Assessment of the water status by the DDI entails its usage independent of the tested cultivars. Because many hundred cultivars or lines may be tested in breeding nurseries, it is not feasible to calibrate a method for measuring the water status for each genotype separately. Therefore, robust optical methods for measuring the water status should not be influenced by miscellaneous traits such as differences in biomass, architecture, pigment concentration and composition that may affect the reflectance measurements of individual cultivars. To assess this assumption, an analysis of variance was conducted including the factors cultivar, DDI and canopy temperature (see Table 1). Canopy temperature was used as a further reference measurement representing actual transpiration of the canopy. The analyses of variance indicated a significant influence of the canopy temperature on the DDI. Therefore, the DDI can be used like surface temperature for measuring drought stress on warm days. One advantage of spectral measurements compared with thermal measurements is, that they are less affected by variable environmental conditions such as radiation.

A significant impact of the cultivar on the DDI index was also detected, which means that at least drought-sensitive and drought-tolerant cultivars could successfully be differentiated by the DDI. No interactive effects of cultivar and canopy temperature on the DDI were observed. The DDI is therefore suited for measuring the water status independent of miscellaneous other cultivar traits.

Accuracy of optical water status measurements

The RWC and canopy temperature show a higher accuracy of measurement than the normalised index and the DDI (see Table 2). The cause for these differences in the measurement accuracy is related to the propagation of errors. Canopy temperature measurement is based on a single parameter, the emission of thermal radiation according to the Stefan-Boltzmann Law. RWC is based on the three parameters fresh weight, dry weight and turgescence weight. The normalised index is based on the measurement of two wavelengths, whereas the DDI is based on four parameters, two wavelengths measured at day and at night. Increasing the number of parameters leads to more potential sources of error, and this can be seen as one cause of the reduced quality of the optical measurements and is a disadvantage of the DDI.

It is also noticeable that the confidence intervals for the optical measurements were smaller in the field trial than in the rain-out shelter trial. This is caused by the smaller plot size and the lower number of single-spot measurements per plot in rain-out shelter trial compared with the field trial. A larger number of measurements per plot leads to a more accurate estimation of the averaged plot reflectance. More consistent plot means are accompanied by smaller confidence intervals for measuring cultivars with three or four replications. Increasing plot size therefore improves the measurement accuracy.

A clear advantage of optical water status measurements is their speed and efficiency. Where destructive measurements of the RWC in a 48-plot field trial required 175 min, optical measurements with a carrier-mounted device could be obtained in seven minutes. This not only reduces the costs for the measurements but also minimises the errors that occur due to diurnal shifts potentially affecting destructively obtained information when measuring for an extended period under changing light and temperature conditions.

Classification of cultivars according to their drought tolerance

To be used as an independent trait representing drought tolerance, the DDI should allow for a differentiation of individual cultivars or a classification of groups of cultivars. The results from the l.s.d. analysis indicated significant differences within the selected cultivars (see Table 4) under warm and sunny conditions and moderate natural drought (rain fed trial on 28 June 2011). A group encompassing drought-tolerant cultivars could be formed including five cultivars characterised by the lowest DDI values. Within this group, the DDI showed no significant
differences. These cultivars could be separated from a group of more drought-sensitive cultivars. The more drought-tolerant cultivars showed only small changes in daytime reflectance and therefore little dehydration. In general, more recently released cultivars such as Eunova showed an improved drought tolerance, while historical cultivars like Heils Franken and Bavaria were revealed to be drought-sensitive. The physiological causes for these differences must be further elucidated. In summary, the developed method allowed the differentiation of groups of genotypes according to drought tolerance.

This work revealed the suitability of indices combining information from the NIR and blue waveband region to retrieve canopy water content information, which is consistent with previously reported findings by Mistlele et al. (2012). Whereas their study favoured a combination of the wavelengths at 780 nm (NIR) and 490 nm (blue), in this work, the combination of 730 nm (red edge) and 457 nm (blue) turned out to fit best for water status measurements. The findings are also in accordance with Bowman (1989) who stated, that the NIR range is suitable for detecting changes in RWC on the leaf level. A previous report indicated the possibility to trace diurnal changes in the water status of plants grown under controlled chamber conditions (Elsayed et al. 2011). In this work, such changes could be detected under field conditions using high-throughput spectral assessments applied to a set of 16 cultivars. Accuracy and repeatability of the measurements proved sufficient for drought stress phenotyping.

An alternative for drought stress testing based on spectral indices, is to statistically integrate the information found in the whole VIS and NIR range. In simplex volume maximisation the similarity of spectra to archetypical spectra of vital respectively senescent plant material is retrieved. Using this approach, drought treatments in pot grown spring barley and irrigated treatments in maize field trials could be distinguished (Römer et al. 2012). An advantage of this approach is that unlike use of spectral indices, no information contained in spectra is lost for drought stress assessment. In contrast, using single bands that are physically connected to distinguishable drought stress symptoms is advantageous. For example drought induced senescence and chlorophyll decomposition can be detected in the visible range (Kipp et al. 2014a), structural changes induced by changes in water status can be detected in the near infrared range (Bowman 1989) and absorption by water molecules and absolute vegetation water content can be detected in the shortwave infrared range (Gao 1996; Ceccato et al. 2002a).

Several recent techniques in remote and proximal sensing have been used for assessing water status and transpiration of single plants and crop canopies. A portable nuclear magnetic resonance (NMR) device was used to measure the diurnal cycle of the water status dynamics of a bean pod (Rascher et al. 2011).

Solar induced chlorophyll fluorescence measured from an aircraft has been used for assessing drought stress in orchards. By this method Zarco-Tejada et al. (2009) could distinguish photosynthetic activity in well watered and drought stressed trees (peach, orange and olive). Remote sensing is also used for assessing the actual evapotranspiration of irrigated crops by solving the surface energy balance based on measurements of shortwave reflectance and thermal emissions. The method has been validated for many crops, including wheat, sunflower, maize and alfalfa (Bastiaanssen et al. 2005). These new techniques have, to our knowledge, up until now not been used for phenotyping large sets of genotypes. Differences in water status of equally watered genotypes are usually smaller than differences across watering treatments. Whether or not these techniques are sufficiently accurate and allow high-throughput phenotyping of genotypes in field or greenhouse trials requires evaluation.

**Relation of drought stress tolerance to yield and other agronomic parameters**

A central goal of crop breeding is improving yield in the target environment for which the cultivar is released. A developed and measured trait should ideally correlate with yield or yield components under drought stress. The DDI expressed significant correlations (Table 5) and negative regression functions (data not shown) with grain yield and yield components such as biomass, harvest index, thousand grain weight and grain number/ear. The DDI measured in a rain fed field trial during grain filling was related to the yield of genotypes that differed in biomass and yield due to drought stress in the late vegetative and early generative phase. Selection for DDI could lead to yield improvements in drought-prone environments. As shown in Fig. 6b, DDI is not directly affected by biomass. The correlation of DDI with final biomass and yield reveals the ability of single genotypes to remain hydrated under moderate drought stress. This enables continuous growth and higher productivity.

In conclusion, it can be stated that (i) a normalised differential spectral index for measuring the water status of barley was systematically retrieved, selecting the wavelengths of 730 and 457 nm; (ii) this index could be applied directly to data measured during daytime, or its diurnal shifts could be used by referencing daytime measurements to pre-dawn measurements. The advantage of this newly developed, referenced diurnal dehydration index (DDI) is, that confounding influences of aerial biomass can be reduced; (iii) a group of drought-tolerant cultivars could be separated from the bulk by applying the DDI; and (iv) the DDI measured during generative growth was significantly correlated with yield and yield components under moderate drought stress under field conditions.

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