Assessing shaded-leaf effects on Photochemical Reflectance Index (PRI) for water stress detection in winter wheat

Xin Yang[1], Shishi Liu[1]*, Yinuo Liu[1], Xifeng Ren[2], Hang Su[1]

[1]School of Resources and Environment, Huazhong Agricultural University, Wuhan, 430070, China
[2]College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China

Correspondence to: Shishi Liu (carol.shishi@gmail.com)

Abstract. The photochemical reflectance index (PRI) has emerged to be a pre-visual indicator of water stress. However, whether the varying shaded-leaf fractions, which may be caused by multiple view angles or the changing crop density in the field, affects the performance of PRI in detecting water stress of crops is still uncertain. This study evaluated the impact of the varying shaded-leaf fraction on estimating relative water content (RWC) across growth stages of winter wheat using seven formulations of PRI. Results demonstrated that for the control treatment the mean PRI of sunlit leaves was slightly higher than those of shaded leaves, but the difference between PRI of sunlit and shaded leaves increased as water resources became more limiting. Despite the difference between PRI of sunlit and shaded leaves, the significance of the linear relationship between RWC and different formulations of PRI did not show obvious variations with shadow fractions, except for the 100% shaded-leaf condition. Among the studied formulations of PRI, PRI based on reflectance at 512 nm as the reference band provided the most accurate estimates of RWC with varying shaded-leaf fractions, except for the 100% shaded-leaf condition. We then applied a uniform RWC prediction model to the data of varying shaded-leaf fractions, and found that the accuracy of RWC predictions was not significantly affected for the mixture of sunlit and shaded leaves. However, RWC estimated with PRI of 100% shaded leaves had the highest RMSE, implying that PRI of the pure shaded leaves may yield inaccurate estimates of plant water status.

1 Introduction

Agriculture consumes about 80%-90% of fresh water worldwide (Gonzalez-Dugo, Durand, and Gastal, 2010). Water stress is one of the most critical abiotic stressors limiting plant growth and crop production (Chaves, Maroco, and Pereira, 2003). Climate change, increasing worldwide shortages of water, and frequent droughts are exacerbating the agricultural water crisis and putting global food security at risk (Hirich et al. 2016; Lei et al. 2016). The assessment of water status in crops is critical for precision irrigation practices, balancing crop production with the water supply and sustainable farming.

Remote sensing provides a unique tool to unobtrusively, efficiently, and quantitatively assess water status in crops. Water stress induces plants' stomatal closure, leading to the increasing leaf temperature due to the decreasing evaporative cooling. Remotely monitoring the change in canopy temperature provides information on instantaneous transpiration status,

and thus thermal remote sensing has been served as an effective tool in detecting water stress for decades (Idso et al. 1981; Sayago, Ovando, and Bocco 2017). However, thermal remote sensing of water stress has limitations in both physiological and operational aspects. The physiological relationship between canopy temperature and stress is not clear for some crops (Villalobos, Testi, and Moreno-Perez 2009). Due to the technical reasons, the spatial resolution of thermal imaging sensors is

5 generally coarser than the visible and infrared sensors, limiting its applications at local scales.

In a recent decade, the photochemical reflectance index (PRI) has emerged to be a pre-visual indicator of water stress. PRI is a normalized difference of reflectance at 531 nm and reflectance at a reference band (e.g. 570 nm) in the visible domain. It is initially proposed as an indicator of the de-epoxidation state of the xanthophyll pigments, relating to photosynthesis (Gamon, Peñuelas, and Field 1992). When the light absorbed by the plants exceeds the photosynthetic

10 demand, de-epoxidation of xanthophyll cycle pigments occurs, leading to the downregulation of photosynthesis (Gamon, Peñuelas, and Field 1992). Numerous abiotic stress, including nutrient deficiency (Shrestha, Brueck, and Asch 2012; Magney et al. 2016), excessive heat (Dobrowski et al. 2005), and water deficit (Muller 2001; Sun et al. 2008; Sarlikioti, Driever, and Marcelis 2010; Zarco-Tejada et al. 2013; Magney et al. 2016), have been shown to trigger the xanthophyll cycle, resulting in the apparent drop in reflectance at 531 nm.

15 As a promising alternative to thermal remote sensing to monitor plant water stress, several previous studies investigate the feasibility of using PRI to assess plant water status at leaf level and canopy level. At leaf level, a number of studies demonstrate a close relationship between PRI and physiological indicators of water stress (Thenot, Méthy, and Winkel, 2002; Shahenshah et al., 2010), but some other studies report a poorer relationship due to the confounding environmental factors (Sarlikioti, Driever, and Marcelis, 2010) or the changes in pigment pools (Sun et al., 2008). At canopy level, studies show

20 stronger correlations between changes in physiological indicators of water stress and PRI, in comparisons with the other indices (e.g. Normalized Difference Vegetation Index (NDVI)) (Suárez et al. 2008; Rossini et al. 2013; Zarco-Tejada et al. 2013). However, the performance of canopy PRI in the water stress detection is affected by canopy structure, canopy cover, and viewing geometry (Rossini et al. 2013; Panigada et al., 2014). Particularly, at seasonal and inter-annual time scales, physiological changes, such as relative water content and pigment pools, concurrently occur with structural changes, such as

25 leaf area index (LAI). Canopy PRI is sensitive to the structural changes during the growth season (Gitelson, Gamon, and Solovchenko, 2017). To minimize the impact of canopy structures on PRI, transformations of PRI are developed using the band insensitive to the canopy structure (Hernández-Clemente et al., 2011), the structural vegetation indices for the normalization (Zarco-Tejada et al., 2013; Gitelson, Gamon, and Solovchenko, 2017), or the radiative transfer modeling results (Hernández-Clemente et al., 2011).

30 PRI is primarily driven by the xanthophyll cycle at the short time scale (e.g. a few hours, two to three days), but shaded leaves may not experience de-epoxidation of xanthophyll cycle as the sunlit leaves do. As PRI is expected to be applied to monitoring water stress at large scale, canopy PRI derived from satellite data includes contributions from both the sunlit leaves and shaded leaves. Hall et al. (2008) and Hilker et al. (2010) found that canopy PRI is strongly dependent on canopy shadow fractions, because the xanthophyll cycle status is affected by incident PAR, which is in turn affected by the level of

self-shading within a canopy. Cheng et al. (2009) examined the contributions of variable sunlit/shaded canopy ratios to the simulation of canopy PRI with the two-layer Markov chain analytical canopy reflectance model, confirming the importance of adding shaded leave in the simulation. Takala and Mõttus (2016) demonstrated that the illumination-induced shadowing effects explained the observed dynamic range of apparent canopy PRI derived from the high spatial resolution airborne imaging spectroscopy data. Zhou et al. (2017) examined PRI of rice leaves and panicles in sunlit and shaded portions of canopies, and they found that the correlations between canopy chlorophyll content and PRI of shaded leaves was much higher than those of sunlit leaves.

Previous studies have shown that within-canopy shadowing effects directly affect PRI of a canopy, but whether the proportion of shaded leaves further influences the performance of detecting water stress in the growth season of a crop using PRI is still uncertain. The objective of this study is to analyze the impact of varying shaded-leaf fractions on the performance of canopy PRI in detecting water stress during the growth season of winter wheat using a hyperspectral imager. To accomplish this objective, we conducted water stress experiments of winter wheat for two consecutive years. Reflectance of shaded and sunlit leaves derived from hyperspectral imagery were mixed with varying fractions to quantify the impacts of shaded leaves on different formulations of PRI in detecting water stress during the growth season.

2 Materials and Methods

2.1. Study site and experimental design

During the growth seasons of 2016 and 2017, two water stress experiments were conducted in the facilities at Huazhong Agricultural University, China (30°28′N, 114°22′E). The mean annual temperature is approximately 17.0 ℃ and the mean annual total precipitation is around 1256 mm. The seeds of cultivar 'Zheng 9023', which is widely planted in central China, were used in the experiment. Seeds were sown on November 2nd, 2015 and November 26th, 2016 respectively, in a rectangular plastic pot (L70cm×W40cm×H35cm) with the density of approximately 250-300 seeds/pot. The soil was silt loam, with the volumetric water content of 26% at the field capacity. Sufficient NPK (5:4:1) fertilizer was applied in the soil before sowing. The experiments consisted of 28 pots in 2015-2016 and 15 pots in 2016-2017. Pest and disease control were conducted in the same time during the growth period, to ensure the plants were not under additional stresses other than different levels of water stress.

Seedlings were growing outdoor under the natural condition before the water stress experiments started. Soil water content was measured every 4-5 days using time domain reflectometry (TDR300, Spectrum Technology Inc., USA), and tap water was supplied if soil water content was below 70% of the field capacity. The water stress treatments started at the end of February, which was the tiller initiation stage. Pots were moved to a rain-out shelter to prevent the external water supply. In 2015-2016, 28 pots were divided into five groups. A group of four pots was used as the control, which had sufficient water supplies throughout the experiment. The other four groups (with six pots for each group) stopped watering on Feb 24th, March 6th, March 28th, and April 8th respectively. In 2016-2017, 15 pots were divided into six groups. A group of three pots

was used as the control, which had sufficient water supplies throughout the experiment. The other four groups (with three pots for each group) stopped irrigation on March 15th, March 22th, March 29th, and April 12th respectively. After irrigation stopped, soils of the treated pots were left to dry to analog the natural drought condition. In 2016, measurements were taken every two to five days depending on the weather conditions until immature senescence occurred. For the water treatment group, three pots of winter wheat were used for capturing hyperspectral images, and the other three pots were used to collect samples. In 2017, measurements were taken every four to six days until immature senescence occurred. For the water treatment groups, one pot of winter wheat was used for capturing hyperspectral images, and the other two pots were used to collect samples. In both years, physiological and spectral measurements were taken in control groups during the whole experiment.

2.2 Physiological measurements

In this study, we used relative water content (RWC) as the indicator of water stress, because RWC was recommended by previous studies as an effective physiological indicator of water status (Hewitt et al., 1985; Siddique, Hamid, and Islam, 2000). We randomly chose three plants in the sampled pot, and top three leaves of the sampled plants were cut from the stem. Leaves were cut into ten small round pieces with a puncher and put into a zip lock bag. Leaf samples were enclosed in a cooler and brought to the laboratory to measure RWC. In the laboratory, fresh weight was measured with an electronic balance. The leaf samples were immersed in distilled water for 16-18 hours. We dried the surface moisture and weighed the turgid weight. Afterward, all samples were put into aluminum boxes to dry in the oven at 105 ℃ for 15-20 minutes, and then dried at 80 ℃ for about 10 hours when a constant dry weight was reached. The RWC of leaf samples was calculated as:

$$RWC = \frac{WF-WD}{WT-WD} \quad (\%) \tag{1}$$

where WF is the fresh weight, WT is the turgid weight, and WD is the dry weight.

2.3 Spectral data

2.3.1 Hyperspectral image acquisition

Hyperspectral images were recorded *in situ* using SOC710VP Portable Hyperspectral Imager (Surface Optics Corporation, USA). The imager has 640×640 pixels and 128 bands in the range of 379-1039 nm, with a spectral resolution of 4.6875 nm and a 25 ° field of view. The transparent shed was open half an hour before measuring began. The imager was set up with a nadir view angle and approximately 1.5 m above the canopy, resulting in the spatial resolution of approximately 1 mm. Hyperspectral images were recorded under sunny and cloudless conditions around midday (12:00-14:00) local time. According to Magney et al. (2016)'s study on white spring wheat, PRI showed pronounced diurnal variations, but PRI measured during peak irradiance, which was approximately between 12:00 and 14:00, showed minimal variations. A reference spectral panel was placed on the pot for each measurement to correct for radiation measurement variations due to differences in solar illumination. The spectral data was acquired by LuCamSoftware Camera Drivers and the HyperScanner

Software platform. After the hyperspectral images acquisition was completed, the radiometric calibration was performed using the SOC's Spectral Radiance Analysis Toolkit (SRAnal), converting the raw DN values of the hyperspectral image to reflectance.

2.3.2 Spectral reflectance extraction and preprocessing

5   We manually selected region of interests (ROIs) of the most deeply shaded leaves and the brightest sunlit leaves in each image using ENVI 5.1 (The Environment for Visualizing Images) of ITT Visual Information Solutions Company (Figure 1). Reflectance within ROIs were averaged and used as reflectance of sunlit and shaded leaves respectively. Based on the assumption of the linear mixture of shadow and sunlit leaves, we mixed different fractions of shaded-leaf reflectance with sunlit-leaf reflectance to evaluate the impact of shaded leaves on detecting water stress with PRI.

10   The derived spectral data was interpolated to 1 nm band width using the cubic spline interpolation function in the MATLAB software. Seven formulations of PRI were calculated for both sunlit leaves and shaded leaves (Table 1). In addition, we calculated the difference (ΔPRI) between PRI of sunlit leaves (PRI_sunlit) and PRI of shaded leaves (PRI_shaded) (Equation (2)).

$$\Delta PRI = PRI\_sunlit - PRI\_shaded \tag{2}$$

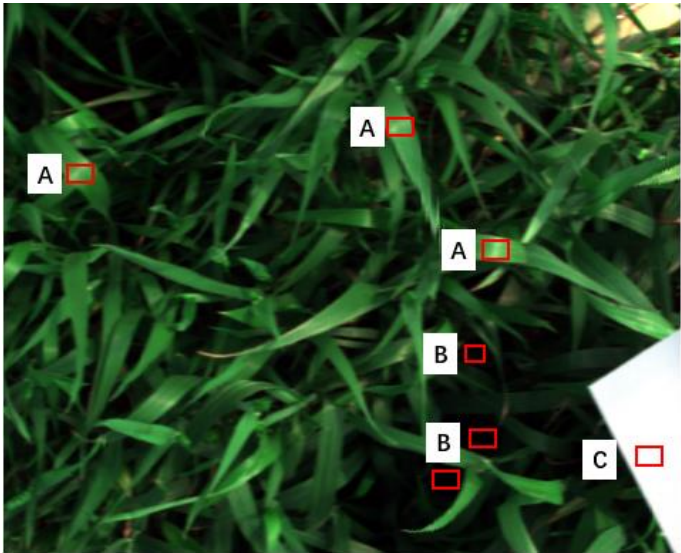



Figure 1. The original hyperspectral image shown in RGB. Region of interests (ROIs) A are the sunlit leaves, ROIs B are the shaded leaves, and ROI C is the reference spectral panel.

20

Table 1. Seven PRI formulations used in this study. R is the reflectance at the specified wavelength in nm.

Index	Equation	Reference
PRI_{570}	(R531-R570)/(R531+R570)	Gamon, Peñuelas, and Field (1992)
PRI1	(R528-R567)/(R528+R567)	Gamon, Filella, and Penuelas (1993)
PRI2	(R539-R570)/(R539+R570)	Penuelas, Filella, and Gamon (1995)
PRI3	(R531-R512)/(R531+R512)	Hernández-Clemente et al. (2011)
PRI4	(R531-R600)/(R531+R600)	Gamon, Filella, and Penuelas (1993)
PRI5	(R531-R670)/(R531+R670)	Gamon, Filella, and Penuelas (1993)
PRI6	RDVI=(R800-R670)/(R800+R670) ^0.5 PRI570/[RDVI*(R700/R670)]	Zarco-Tejada et al. (2013)

2.4. Statistical Analysis

Measurements taken from pots that had the same water treatments were averaged and used in the analysis. The maximum, minimum, coefficient of variation (CV), and standard deviation were used to describe the range and the variation of observations. To analyse the variations of PRI of sunlit and shaded leaves during the water stress treatment, we divided all the data into seven groups according to RWC values (RWC between 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.5-0.6, 0.6-0.7, 0.7-0.8, and >0.8). The mean and the standard deviation were calculated to evaluate the variations of PRI against RWC. The Pearson correlation coefficient (r) measured the linear correlation between RWC and PRI calculated by reflectance of varying ratio of sunlit to shaded leaves. The least-square linear regression model was established to estimate RWC with PRI of sunlit leaves and shaded leaves. R^2 was used to evaluate the significance of the linear regression model, and the root mean square error (RMSE) was used to measure the actual average differences between measurements and modelled predictions. Statistical analyses were performed in the MATLAB (R2011a) software.

3 Results

3.1 PRI of sunlit leaves and shaded leaves

The spectra of sunlit leaves and shaded leaves are presented in Figure 2. The reflectance of the shaded leaves was lower than that of the sunlit leaves. Unlike the spectra of sunlit leaves, the peak in the green region of shaded leaves was not obvious.

We calculated the difference (△PRI) between PRI of sunlit leaves and shaded leaves for the control treatment and water stress treatment separately. For both the control treatment and water stress treatment, the positive mean value of △PRI indicated that PRI of sunlit leaves were higher than those of shaded leaves, but the mean △PRI was much larger for the water stress treatment. Take PRI570 as an example, PRI570 of sunlit leaves and shaded leaves declined as water resource

became limiting after irrigation stopped. △PRI570 became pronounced at RWC smaller than 0.5, and △PRI570 was minimized at RWC higher than 0.8.

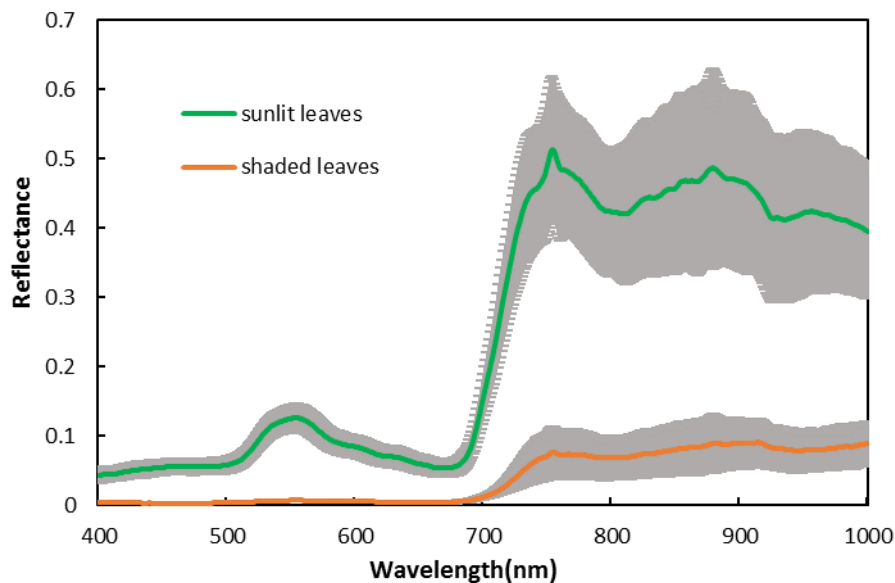



Figure 2. Spectra of sunlit and shaded leaves. The solid lines are the mean reflectance of the samples and the gray shade are the standard deviation.

Table 2. The maximum, minimum, mean, CV and range of the difference (△PRI) between PRI of sunlit leaves and PRI of shaded leaves for the control treatment (**a**) and the water stress treatment (**b**).

(**a**)

	Maximum	Minimum	Mean	CV	Range
△PRI570	0.0519	-0.0178	0.0192	1.1877	0.0696
△PRI1	0.0425	-0.0287	0.0106	2.1840	0.0713
△PRI2	0.0585	-0.0042	0.0227	0.7684	0.0627
△PRI3	0.0976	-0.0565	0.0047	0.9691	0.1541
△PRI4	0.1413	-0.0041	0.0643	0.6256	0.1454
△PRI5	0.3075	0.0327	0.1698	0.5289	0.2748
△PRI6	0.1549	-0.0152	0.0515	1.0431	0.1701

(b)

	Maximum	Minimum	Mean	CV	Range
△PRI570	0.1050	-0.0170	0.0441	0.8095	0.1220
△PRI1	0.0835	-0.0172	0.0253	1.1064	0.1007
△PRI2	0.1109	-0.0192	0.0519	0.7221	0.1301
△PRI3	0.1660	-0.1035	0.0374	1.5302	0.2695
△PRI4	0.2608	-0.0398	0.1362	0.6363	0.3006
△PRI5	0.6119	-0.0061	0.3084	0.5196	0.6180
△PRI6	1.3637	-0.0352	0.2596	1.2326	1.3989

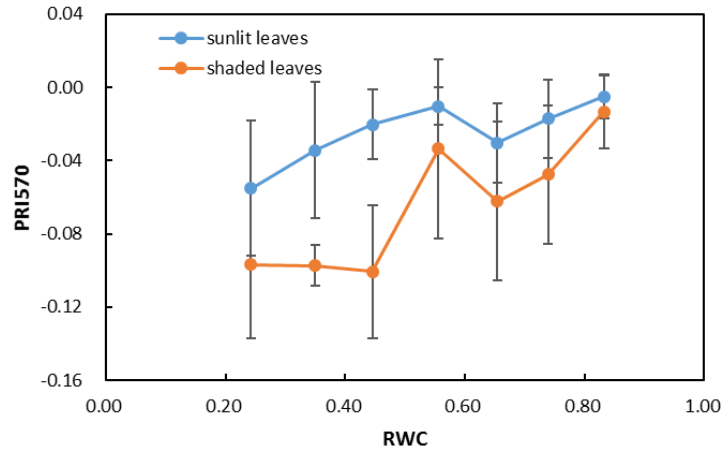



Figure 3. Variations in PRI570 of sunlit leaves and shaded leaves against RWC. Data were divided into seven groups according to RWC values (RWC between 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.5-0.6, 0.6-0.7, 0.7-0.8, and >0.8).

3.2 The impact of shaded leaves on water stress detection

To assess the impact of shaded leaves on detecting water stress with PRI, we mixed different fractions of shaded-leaf reflectance with sunlit-leaf reflectance, and analyzed the relationship between RWC and PRI calculated from the mixed reflectance of shaded and sunlit leaves. Different formulations of PRI were all positively correlated with RWC (Table 3). Among the studied formulations of PRI, PRI3 showed the strongest correlation with RWC in winter wheat with varying shaded-leaf fractions, except for 100% shaded-leaf condition. The weaker correlation with RWC for the 100% shaded-leaf condition can be also found for PRI6. Whereas for the other formulations of PRI, the correlation coefficients between RWC and PRI did not show pronounced differences with varying shaded-leaf fractions. Figure 4 illustrated examples of the significant relationships between RWC and PRI of sunlit leaves and shaded leaves, respectively. PRI2 and PRI3 sunlit leaves had strong correlation with RWC, but those of shaded leaves were weakly correlated with RWC.

We further analyzed the impact of shaded-leaf fractions on the slope and intercept of the linear regression model between PRI and RWC (Table 4). The slope and intercept of PRI1, PRI2, PRI4, and PRI6 were strongly correlated with fractions of shaded leaves (Table 4). The intercept of the linear regression model between PRI570 and RWC was weakly correlated with fractions of shaded leaves, but the slope was strongly correlated with fractions of shaded leaves (Figure 5).

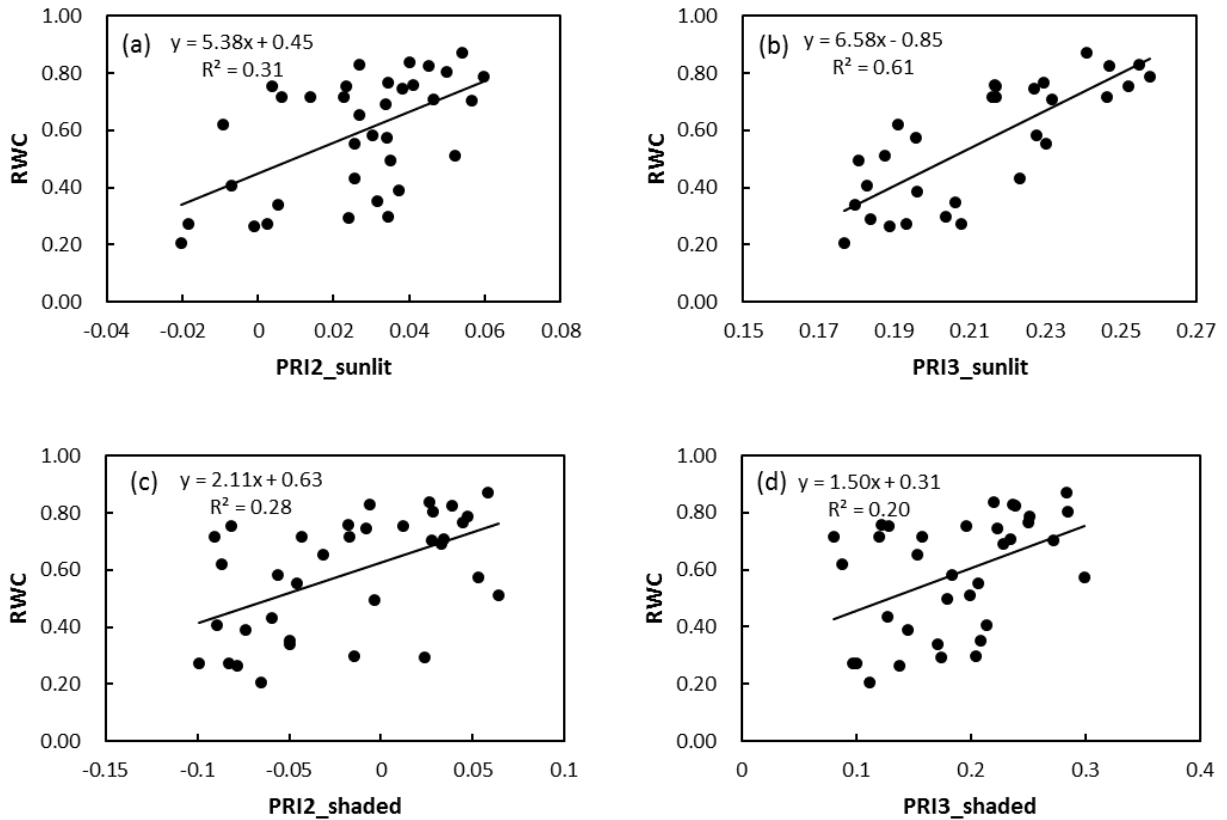
5 The values of slope and intercept of the linear regression model of PRI3 remained almost stable with the varying shaded-leaf fractions, except for the condition of 100% shaded leaves (Figure 5).

10 **Figure 4.** Examples of the linear relationship between PRI of sunlit leaves (PRI2 (**a**), PRI3 (**b**)) and RWC, and the linear relationship between PRI of shaded leaves (PRI2 (**c**), PRI3 (**d**))and RWC.

Table 3. Pearson correlation coefficients between RWC and different formulations of PRI calculated by reflectance of varying ratio of sunlit leaves to shaded leaves in winter wheat during the study period.

RWC	Shaded 0% sunlit100%	Shaded 10%sunlit 90%	Shaded 20%sunlit 80%	Shaded 30%sunlit 70%	Shaded 40%sunlit 60%	Shaded 50%sunlit 50%	Shaded 60%sunlit 40%	Shaded 70%sunlit 30%	Shaded 80%sunlit 20%	Shaded 90% sunlit10%	Shaded 100% sunlit0%
PRI570	0.42*	0.42*	0.43**	0.44**	0.45**	0.47**	0.48**	0.50**	0.53**	0.56**	0.55**
PRI1	0.26	0.26	0.27	0.28	0.30	0.32	0.34*	0.38*	0.43*	0.50**	0.58**
PRI2	0.56**	0.56**	0.56**	0.57**	0.58**	0.58**	0.59**	0.60**	0.60**	0.59**	0.53**
PRI3	0.78**	0.79**	0.80**	0.80**	0.81**	0.82**	0.82**	0.82**	0.79**	0.70**	0.44**
PRI4	0.54**	0.54**	0.55**	0.56**	0.57**	0.58**	0.58**	0.59**	0.59**	0.58**	0.52**
PRI5	0.16	0.18	0.20	0.22	0.24	0.27	0.30	0.33	0.36*	0.38*	0.34*
PRI6	0.47**	0.48**	0.48**	0.48**	0.48**	0.49**	0.49**	0.48**	0.46**	0.41*	0.19

**. Correlation coefficient significant at $P<0.01$.

*. Correlation coefficient significant at $P<0.05$.

Table 4. Relationships between shaded leaves fractions and the slope and intercept of the linear regression equation between PRIs.

	PRI570	PRI1	PRI2	PRI3	PRI4	PRI5	PRI6
Slope	-0.38	0.70 *	-0.65 *	-0.54	-0.65 *	-0.31	-0.69 *
Intercept	0.96 **	0.93 **	0.84 **	0.60	0.83**	0.73*	0.88 **

**. Correlation coefficient significant at $P<0.01$.
*. Correlation coefficient significant at $P<0.05$.

To evaluate if these changes in the values of linear regression parameters affected the accuracy of RWC estimates, we applied the linear regression model derived from the PRI of the generally applicable sunlit leaves/shadow ratio of 50/50 to detect water stress using PRI of the varying sunlit leaves/shadow ratio. Results showed that RMSE of RWC estimates did not vary significantly with shaded-leaf fractions, but RWC estimated with PRI of 100% shaded leaves had the highest RMSE, implying that PRI of the pure shaded leaves may yield inaccurate estimates of plant water status.

Table 5. RMSE of RWC estimated by the linear regression model derived from the PRI of the generally applicable sunlit leaves/shadow ratio of 50/50, using PRI calculated with reflectance of varying ratio of sunlit leaves to shaded leaves in winter wheat during the study period.

	Shaded 0% sunlit100%	Shaded 10%sunlit 90%	Shaded 20%sunlit 80%	Shaded 30%sunlit 70%	Shaded 40%sunlit 60%	Shaded 60%sunlit 40%	Shaded 70%sunlit 30%	Shaded 80%sunlit 20%	Shaded 90% sunlit10%	Shaded 100% sunlit0%
PRI570	0.1826	0.1820	0.1813	0.1804	0.1794	0.1768	0.1753	0.1743	0.1783	0.2366
PRI1	0.1980	0.1974	0.1968	0.1960	0.1950	0.1920	0.1897	0.1865	0.1825	0.1935
PRI2	0.1711	0.1705	0.1699	0.1691	0.1683	0.1666	0.1662	0.1680	0.1803	0.2786
PRI3	0.1584	0.1578	0.1571	0.1563	0.1555	0.1539	0.1539	0.1567	0.1720	0.2808
PRI4	0.1747	0.1738	0.1728	0.1717	0.1706	0.1683	0.1683	0.1718	0.1902	0.3037
PRI5	0.1990	0.1982	0.1973	0.1964	0.1953	0.1933	0.1929	0.1949	0.2061	0.2772
PRI6	0.1962	0.1959	0.1956	0.1953	0.1949	0.1939	0.1931	0.1918	0.1908	0.2346

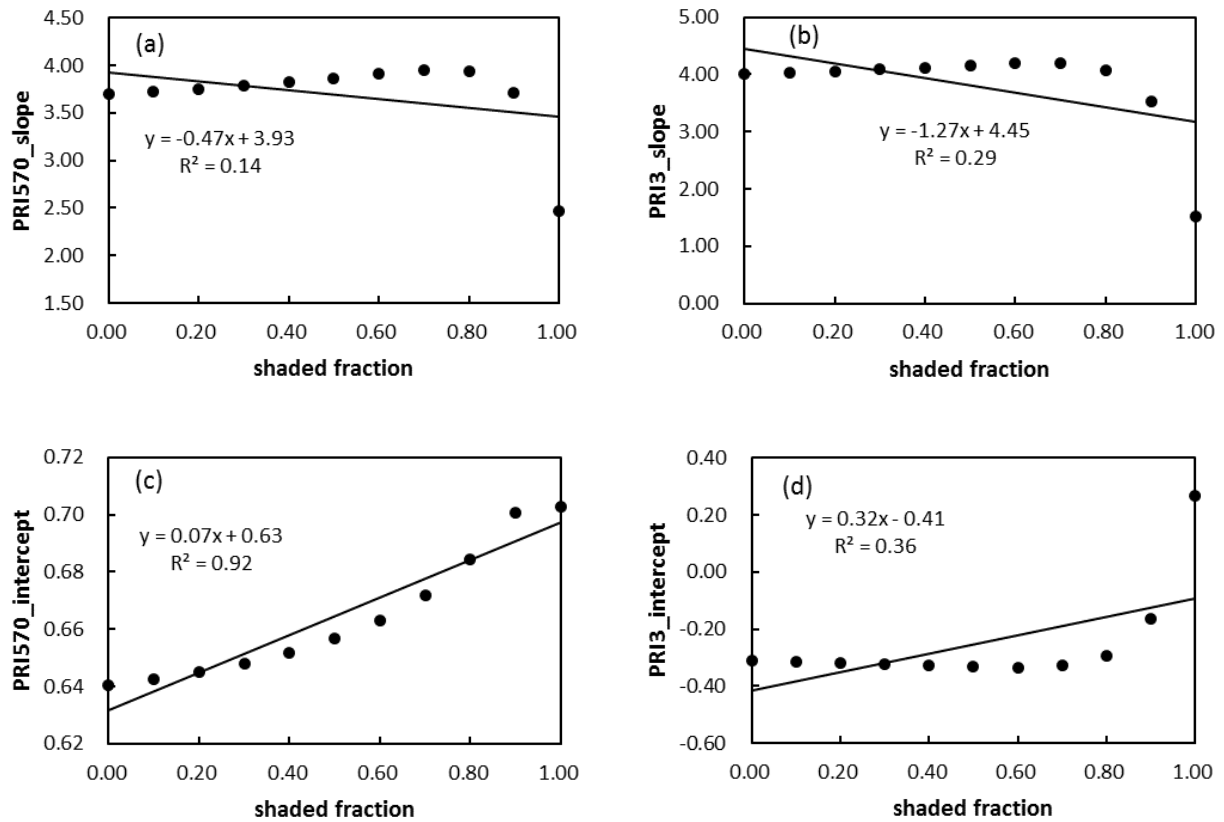



Figure 5. Relationships between shaded-leaf fractions and the slope of the linear regression equation of RWC and PRI570 (**a**), PRI3 (**b**); and relationships between shaded-leaf fractions and the intercept of the linear regression equation of RWC and PRI570 (**c**), PRI3 (**d**).

4. Discussion

Theoretically, sunlit leaves are more likely to experience high light-induced environmental stress than shaded leaves (Hilker et al., 2008; Middleton et al., 2009; Cheng et al., 2012). Data from previous field samplings and the model simulations, although limited, confirmed the impact of shaded-leaf fractions on PRI values (Middleton et al., 2009; Cheng et al., 2012; Takala and Mõttus, 2016). While interests of detecting water stress of plants with PRI are increasing, studies rarely analyzed the impact of shaded leaves on the performance of PRI in water stress detection. This study quantified the differences between PRI of sunlit and shaded leaves in winter wheat under control and water stress conditions, and

investigated the impact of varying shaded-leaf fractions on water stress detection during the growth season, using different formulations of PRI derived from hyperspectral images.

Our results showed that for the control treatment the mean PRI of sunlit leaves was slightly higher than those of shaded leaves. Take PRI570 for example, ΔPRI570 ranged from -0.0178 to 0.0519, roughly agreed with results presented in previous studies. Takala and Mõttus (2016) reported the range of ΔPRI without a shadow correction was -0.01 – 0.10 at the boreal forest. Middleton et al. (2009) reported ΔPRI of -0.035 at a Douglas-fir forest in Canada. Cheng et al. (2012) demonstrated that the average PRI values varied from -0.008 to 0.005 for sunlit leaves and from 0.002 to 0.022 for shaded leaves measured in the corn field. Mõttus et al. (2015) presented the difference between canopy PRI (including PRI of shaded leaves) and PRI of sunlit leaves ranged from -0.025 to 0.073 for pine, spruce and birch. In summary, both positive values and negative values of ΔPRI570 can be found in previous studies (Hilker et al., 2008; Middleton et al., 2009; Cheng et al., 2012), but the difference between PRI of sunlit leaves and those of shaded leaves was small for healthy vegetation.

Interestingly, our results showed that △PRI was much higher for the water stress treatment than the control treatment. △PRI increased as water resources became more limiting, but it then decreased when prolonged drought caused premature senescence. The increased △PRI might be due to the more sever chlorophyll degradation on old leaves, induced by a sustained water stress deficit (Bolhar-Nordenkampf, Hofer, and Lechner, 1991; Ciganda, Gitelson, and Schepers, 2012; Liu et al., 2015). As several studies proved that PRI was related to the pigment content (Suárez et al., 2009; Gitelson, Gamon, and Solovchenko, 2017), the early degradation of chlorophyll content in the bottom shaded leaves may influence their photosynthetic potential, and thus lead to the nonsynchronous change of PRI values between the top sunlit leaves and the bottom shaded leaves. The weaker correlation between PRI of shaded leaves and RWC (Figure 5) also supported the hypothesis of the nonsynchronous change of PRI values between the sunlit and shaded leaves. Both the sunlit and shaded leaves eventually wilt after the prolonged water stress, resulting in the decreased range in △PRI at the end of the water stress treatment.

Although the PRI of shaded leaves were different from PRI of sunlit leaves under both control and water stress conditions, the effect of the varying fractions of shaded leaves did not lead to the substantial change in the significance of the relationship between PRI and RWC. We hypothesized it was because the shallow soil in the pot experiment caused the quick wilting during the water stress treatment, and thus the changes in leaf area and pigment content intertwined with physiological responses. Among the studied formulations of PRI that were proved to minimize the effect of the structural change in canopies in previous studies (Hernández-Clemente et al., 2011; Zarco-Tejada et al., 2013), PRI3 that used reflectance at 512 nm as the reference band provided the most accurate estimates of RWC with varying shaded-leaf fractions, except for the 100% shaded-leaf fraction. PRI3 was originally developed for the needle tree based on the evidence that reflectance at 512 nm was not responsive to the change in xanthophyll epoxidation state (Hernández-Clemente et al. 2011). In their study, PRI3 showed the highest correlation with the stomatal conductance and water potential at the canopy level, and the lowest sensitivity to canopy structure, in comparison with PRI570 and NDVI.

Our results also showed the superior performance of PRI3 than the other formulations of PRI in estimating RWC, implying that for winter wheat band 512 nm might be a better reference band that could maximize the physiological responses of band 531 nm. Unfortunately, we could not provide direct evidence of PRI3's superior sensitivity to the change in xanthophyll epoxidation state, due to the lack of measurements of the xanthophyll epoxidation state and leaf

5   area index.

Magney et al. (2016) used the difference between the midday PRI and early morning PRI (PRI_0) to disentangle the facultative (diurnally changing) and constitutive (seasonally changing) components of the PRI signal, based on the assumption that the early morning PRI represented a 'steady-state' prior to xanthophyll cycle deepoxidataion. They found the stronger seasonal responses of PRI- PRI_0 to vapor pressure deficit, suggesting that PRI- PRI_0 was mainly

10   facilitated by short term changes in the xanthophyll cycle as opposed to longer-term pigment changes. Hwang et al. (2017) found that the ratio (sPRI) of sunlit canopy PRI (backward direction images) to shaded canopy PRI (forward direction images) captured drought signals in a temperate deciduous forest. Inspired by these studies, we tried to use ΔPRI and PRI_sunlit/PRI_shaded to disentangle the facultative and constitutive components. However, the correlation between RWC and ΔPRI or PRI_sunlit/PRI_shaded was not significant, and thus results were not shown in the

15   manuscript.

5. Conclusion

This study evaluated the impact of the varying shaded-leaf fraction on seasonal water stress detection in winter wheat using different formulations of PRI. Results demonstrated that for the control treatment the mean PRI of sunlit leaves was slightly higher than those of shaded leaves, but the difference between PRI of sunlit and shaded leaves increased as water

20   resources became more limiting. Despite the difference between PRI_shadow and PRI_leaf, the significance of the linear relationship between RWC and different formulations of PRI did not show obvious variations with shadow fractions, except for the 100% shaded-leaf condition. Among the studied formulations of PRI, PRI3 based on reflectance at 512 nm as the reference band provided the most accurate estimates of RWC with varying shaded-leaf fractions, except for the 100% shaded-leaf condition. Furthermore, we applied the linear regression model derived from the PRI of the generally applicable

25   sunlit leaves/shadow ratio of 50/50 to detect water stress using PRI of the varying sunlit leaves/shadow ratio, and found that the accuracy of RWC estimates did not vary significantly with shaded-leaf fractions, but RWC estimated with PRI of 100% shaded leaves had the highest RMSE, implying that PRI of the pure shaded leaves may yield inaccurate estimates of plant water status. This study provides useful information on remote detection of water stress for accurate irrigation scheduling and yield forecast. Further research is indeed needed to understand the shaded-leaf effect on PRI and water stress detection,

30   especially for crops have different canopy geometry from winter wheat.

Author Contributions. Xin Yang participated in the data collection, conducted data pre-processing and statistical analysis, and wrote methods and results sections. Dr. Shishi Liu designed the experiment with Dr. Xifeng Ren, participated in the data collection, and wrote introduction, discussion, and conclusion sections. Yinuo Liu, Xifeng Ren, and Hang Su participated in the data collection, and provided valuable suggestions to the manuscript.

Competing interests. The authors declare no conflict of interest.

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