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Fluorescence-based sensing of drought-induced stress in the vegetative phase of four contrasting wheat genotypes

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ABSTRACT

The aim of this study was to analyse drought-induced changes of the blue (BF), green (GF), and far-red (FR) fluorescence of two *T. aestivum* (Sumo and Tulsa) and two *T. durum* (Trinakria and Creso) wheat cultivars; Sumo and Trinakria have previously been characterised as comparatively drought tolerant. As a result of water deficit, the BF, GF, and FR fluorescence intensities and several fluorescence ratios significantly changed in these cultivars when grown under greenhouse and climate chamber conditions. However, the observed modifications were partially reversible, and, in most cases, the re-watering of drought stressed plants caused the fluorescence signals to approach the values of the control plants. The most robust fluorescence index to indicate drought stress was the UV-excited blue-to-far-red fluorescence ratio (BFRR), which significantly increased irrespective of the wheat cultivar and the physiological age of the tissue. The reduction of the UV-induced FR fluorescence, which was associated with leaf shrinkage, the reduction of the chlorophyll content, and the increase in flavonols in the epidermis was responsible for the increase of BFRR. The cultivars previously classified as more tolerant to drought (Sumo and Trinakria) had a stronger BFRR modification compared to the sensitive cultivars (Tulsa and Creso). Thus, we conclude that drought-induced stress in the vegetative phase can be rapidly and non-destructively sensed with multiparametric fluorescence devices. Due to their robustness, multiparametric fluorescence-based indices also have a large potential to support the in-field characterisation of the drought tolerance of genotypes. Furthermore, the short-term modification of the indices after drought and re-watering reveal the potential of these parameters as additional tool for crop management.

1. Introduction

With global climate change, the scarcity of water for agronomic purposes will become the most important issue for crop production (Hura et al., 2007; Pennisi, 2008; Saint Pierre et al., 2012). From an agronomical point-of-view, drought stress is a situation in which the limited water supply prevents the growth or yield of a plant from reaching the genetic potential while exceeding the plants' capacity of homeostatic mechanisms to compensate with the deficit. Thus, one major adaptation strategy for plant production relies on the screening and cultivation of stress-tolerant cultivars to maintain high productivity and quality standards with less water (Dodd et al., 2011; Gregory and George, 2011).

Historically, the response of plants to water deficit has been evaluated based on genetic, biochemical, and morpho-physiological traits. Among others, the leaf gas exchange, water content (WC), relative WC, leaf water potential, osmotic potential, chlorophyll and proline concentrations, and regulation of the electron transport have been used as indicators (Fischer and Maurer, 1978; Dib et al., 1994; Araus et al., 1998; Loboda, 2000; Siddique et al., 2000; Golding and Johnson, 2003; De Leonardis et al., 2007; Hura et al., 2007; Maccaferri et al., 2011). Furthermore, it has been proposed that parameters, such as the total yield (Fischer and Maurer, 1978), photosynthesis, transpiration and water-use efficiency (Blum, 2005), the effective use of water and the canopy temperature (Blum et al., 1989), the crop water stress index (Alderfasi and Nielsen, 2001) and the water stress index (Rizza et al., 2004) can be used to select genotypes that are tolerant to

Abbreviations: A, assimilation; BF, blue fluorescence; BFRR_UV, blue-to-far-red emission ratio; BGFR_UV, blue-to-green fluorescence emission ratio; Chl, chlorophyll index; DAS, days after sowing; ETR, electron transport rate; FLAV, flavonol index; FRF, far-red fluorescence; GF, green fluorescence; LED, light emitting diodes; LWC, leaf water content; NPQ, non-photochemical quenching; PAR, photosynthetic active radiation; qp, photochemical quenching; RWC, relative water content; WD, water deficit; WDRW, water deficit and re-watered.

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drought. However, the responses to water deficit may change at the different stages of growth and maturity of the crop (Sullivan and Eastin, 1974). Therefore, robust parameters are required to assess drought tolerance, particularly when physiological evaluations and genotype screening are performed at the vegetative stages of development.

The crop response to water deficit is strongly influenced by the time, intensity, duration and frequency of the stress and by diverse plant-soil-atmosphere interactions (Saint Pierre et al., 2012), which are also related to the complexity and the dynamic nature of drought events (Berger et al., 2010). Under natural conditions, neither the frequency nor the intensity of drought is reproducible over several years and different locations (Grzesiak et al., 2007; Hura et al., 2007). Alternatively, experiments in greenhouses or phytotrons offer more standardised conditions. Additionally, modern high-throughput phenotyping approaches require realistic experimental protocols to identify high-performance lines (Saint Pierre et al., 2012; Tuberosa, 2012).

The interest in selecting high-performance lines of bread wheat (Fleury et al., 2010) and *durum* wheat (Schuhwerk et al., 2010; Maccaferri et al., 2011) is well documented. Based on morpho-physiological traits monitored using non-destructive imaging technologies, which are associated with superior feature extraction and data analysis, high-throughput phenotyping stations deliver a complete picture of the plant and the modifications during its lifetime (Berger et al., 2010). However, phenotyping has become the major cost- and rate-limiting step in the genetic analysis of drought tolerance (Fleury et al., 2010). Within this context, the development of rapid and affordable procedures to characterise components of the drought response will be critical for improving genetic resolution (Fleury et al., 2010). Thus, the establishment of flexible and non-destructive methods for the reliable detection of the physiological responses of plants under different growth environments and agronomical conditions would strongly support the evaluation of genotypes under real conditions.

The advantages of fluorescence-based methods to evaluate the physiological response of plants to stress are well described (Dahn et al., 1992; Buschmann et al., 2000; Lenk et al., 2007). In particular, chlorophyll fluorescence, which is influenced by the functionality of the photosystems, the electron flow and chlorophyll concentration, has been used to identify constrains in plant physiology (Havaux and Lannoye, 1985; Baker and Rosenqvist, 2004; Buschmann, 2007; Baker, 2008). For this purpose, the Pulse-Amplitude-Modulated (PAM) chlorophyll fluorescence is a widely adopted technique, even though the ideal measurement conditions involving dark–light transitions imposes some practical limitations. Alternatively, blue and green fluorescence as the absolute intensity or as related to the red or far-red fluorescence intensity might provide reliable information about the stress-induced accumulation of fluorescing compounds in the epidermis (Cerovic et al., 1999; Hura et al., 2009). Therefore, this type of fluorescence is usually used as a simple or a complex ratio that reduces the interference of factors that are not directly related to the experiment (e.g., equipment type, measuring temperature, distance between sensor and sample). Furthermore, depending on the excitation light, these ratios give more precise information about the type and localization of the pigments in the samples (Bilger et al., 1997; Cerovic et al., 1999).

Accordingly, portable optical sensors have been developed to record the fluorescence intensity in defined spectral bands, e.g., blue, green, red and far-red, after excitation with different light sources (Ben Ghozlen et al., 2010). Thus, it is possible to estimate the content of chlorophyll, anthocyanins and flavonols based on the absolute fluorescence intensities, simple and complex fluorescence ratios, and light transmission (Goulas et al., 2004; Cerovic et al., 2005; Morales et al., 2011; Cerovic et al., 2012). The potential of specific indices obtained using the Multiplex® and Dualex® devices was demonstrated for the monitoring of the maturation of apples (Betemps et al., 2012), olives (Agati et al., 2005) and grapes (Ben Ghozlen et al., 2010; Bramley et al., 2011; Baluja et al., 2012), also in the scope of precision agriculture. However, their potential use for sensing abiotic and biotic stresses has not yet been fully exploited; for this, it is necessary to identify in advance the appropriate indices and to validate them using different genotypes grown under different conditions.

In our approach, we selected two *Triticum aestivum* and two *Triticum durum* cultivars for water-deficit studies under greenhouse and climate chamber conditions. We selected one tolerant and one sensitive cultivar for each *Triticum* species. In our experiments, we recorded the blue, green and far-red fluorescence of leaves at different physiological ages. Our work was based on the hypothesis that cultivars with a similar tolerance degree may present a similar drought-induced modification of fluorescence patterns in the vegetative phase. As a reference approach, we assessed the assimilation rate using combined chlorophyll fluorescence measurements during the water-deficit and re-watering phases.

2. Materials and methods

2.1. Plant material

Experiments were performed with two *T. aestivum* and two *T. durum* cultivars. The *T. aestivum* cultivars, Sumo and Tulsa (Saaten Union Recherche SAS, France), were selected on the basis of their physiological response to water deficit in pot experiments. Accordingly, Sumo was classified as more tolerant to drought stress than Tulsa (*personal communication*, Prof. Dr. Wolfgang Friedt, University of Gießen, Germany). The *T. durum* cultivars, Trinakria and Creso (courtesy of Agricultural Research Council, Genomics Research Centre, CRA-GPG, Italy), were characterised under field conditions as tolerant and susceptible to water deficit, respectively, classifications that were based on specific physiological parameters, reflectance measurements, and grain yield (Flagella et al., 1992; Gavuzzi et al., 1993; De Leonardis et al., 2007).

2.2. Experimental conditions

The experiments were conducted under greenhouse and climate chamber conditions. The seeds were sown in pots (13 cm × 13 cm, volume 1 L) filled with decomposed raised-bog-peat soil (Floradur B, Floragard Vertrieb GmbH, Oldenburg, Germany). The pots were placed on trays to enable sub-irrigation. The plants were fertilised once a week with a commercial fertiliser solution (Hydrokani® C2, HydroAgri, Neuilly-sur-Seine, France) supplemented with iron (Sequestrene® 138Fe100SG, Syngenta Agro, St Cyr l'Ecole, France). In the greenhouse, the maximum photosynthetic active radiation (PAR) ranged between 214 and 260 μmol photon m^{-2} s^{-1}; the average day/night temperature was 22/12 °C. The plants for the climate chamber experiments were grown under similar conditions. The plants were transferred from the greenhouse to the climate chamber (day length 16 h; PAR 195–363 μE m^{-2} s^{-1}; day/night temperature 22/15 °C) when the third leaf emerged (15 days after sowing, DAS).

2.3. Water deficit and re-watering

Water deficits was initiated in the corresponding treatments (WD = water deficit) when the third leaf attained 3–4 cm in length (17-18 DAS). For this purpose, the irrigation was ceased, causing a progressive water deficit in contrast to the well-watered control plants. The water withdrawal was maintained until 23 DAS (greenhouse) and 27 DAS (climate chamber); the plants previously

exposed to water deficit were then re-watered (WDRW = water deficit, re-watered). The experiments were completed two days after re-watering the plants in the greenhouse (25 DAS) and climate chamber (29 DAS).

2.4. Sensors and steps for data acquisition

2.4.1. The Multiplex® sensor

Multiplex®3 (FORCE-A, Orsay, France) is a hand-held multiparameter fluorescence sensor that operates with light-emitting diodes (LEDs) for excitation and filtered photodiodes for fluorescence detection, as described elsewhere (Ben Ghozlen et al., 2010). In our study, we used a slightly modified version of the commercial sensor. The standard commercial version of the sensor has a four-colour excitation and three detector channels. It has six LED-matrix UV light sources peaking at 375 nm and protected by 377 nm filters (Semrock, Rochester, NY, USA) and three red–green–blue (RGB) LED matrices emitting light at 470 nm (blue, B), 516 nm (green, G) and 635 nm (red–orange, R) protected by 650 nm short-pass filters (Asahi Spectra, Torrance, CA, USA). The LEDs are pulsed sequentially at 240 Hz at 45 μs per flash. For the present study, the three synchronized photodiode fluorescence detectors were protected by the following interference filters (Intor, Socorro, NM, USA): 450WB40 for blue fluorescence (BF), 531WB40 for green fluorescence (GF) and 750WB65 for far-red fluorescence (FRF). In addition, the FRF channel had a 3 mm RG715 long-pass glass filter (Schott, Mainz, Germany). Blue and green excitation was turned off. A special diaphragm was mounted in front of the sensor to illuminate only a 4-cm-diameter surface (12.6 cm^2) at a 10-cm distance from the sources and detectors. Each measurement consisted of a train of 250 flashes of two colours (UV and R). The sensor-specific software calculated a set of ratios after each series of two-colour flashes.

In addition to the extensive information provided by the equipment (absolute fluorescence intensities after excitation with different lights and many ratios among single signals), we focused our data analysis and interpretation on those indices having a strong link to drought stress physiology. For this purpose, we selected the blue-to-far-red fluorescence emission ratio under UV excitation (BFRR_UV), the blue-to-green fluorescence emission ratio under UV excitation (BGFR_UV), and the FLAV index, as indicated in Table 1.

2.4.2. The Dualex® sensor

The Dualex Scientific® sensor is a hand-held leaf-clip sensor designed to non-destructively evaluate the content of chlorophyll and epidermal flavonols (Cerovic et al., 2012). The determination of the leaf chlorophyll content, expressed in μg per cm^2 units, is based on the measurement of the difference in the transmission of two distinct wavelengths, one close to red and one in near infrared (Cerovic et al., 2012). The measurement of the UV optical absorbance in the epidermis is based on the fluorescence emitted by the chlorophyll located in the mesophyll and is directly linked to the concentration of polyphenols (flavones in the case of wheat) in the epidermis. The principle of this screening method is described elsewhere (Goulas et al., 2004). We used the chlorophyll index (Chl), the flavonol index (FLAV) and the nitrogen balance index (NBI), which is based on the ratio between the mesophyll chlorophyll and epidermal flavone leaf contents (Cartelat et al., 2005).

2.5. Sequential steps for data acquisition

To address the impact of the treatments, we chose leaf two (L2), three (L3) or four (L4) in the case of the greenhouse plants and L3 for the climate chamber plants. The Dualex® and Multiplex® measurements were done sequentially at the adaxial side of detached leaves. Next, the leaves were scanned with a commercial scanner (CanonScan LiDE 90, Canon, France), and the leaf width was determined at the measuring spot using the freeware 'Image J' (version 1.43, National Institutes of Health, USA). The fresh weight (FW) and the dry weight (DW; oven dried for 48 h at 50 °C) of the leaves were used to calculate the leaf water content (LWC) according to the equation

$$\mathrm{LWC} = \frac{(\mathrm{FW} - \mathrm{DW})}{\mathrm{FW}} \quad (1)$$

2.6. Photosynthesis, chlorophyll fluorescence and relative water content

In a separate experiment, the photosynthetic activity of the *T. aestivum* cultivars Sumo and Tulsa was evaluated under well-watered and water-deficit conditions. The plants were grown in the greenhouse, and both the watering and fertilisation were as described above. Twenty-three days after sowing, when L3 was fully developed and L4 had already emerged, the water supply was stopped, and monitoring of the control and water-deficit treatment groups was initiated. The measurements were conducted until 31 DAS when the plants were re-watered and allowed to recover. On the next day, the final photosynthesis was measured after the recovery process.

The determination of the leaf gas exchange was coupled with measurements of modulated chlorophyll fluorescence using a portable open gas-exchange system (LI-6400-40; LI-COR, Inc., Lincoln, NE). For each measurement, three leaves were fixed with the middle portion in the chamber such that the effective area of the chamber was covered without the overlapping of the leaves. The measurements of the control leaves revealed the genotype-specific characteristics. The fluorescence kinetics of dark-adapted leaves with the associated A/Ci (assimilation versus internal CO_2) curves, or light–response curves, were obtained daily from DAS 23 to DAS 32. All the measurements were performed at 25 °C. The fluorescence kinetic of dehydrated leaves was analysed daily, beginning at 25 days after sowing. The recordings of the fluorescence kinetics on the control leaves were performed at a constant CO_2 concentration

Table 1
Nomenclature of Multiplex® signals used in this study. (A) The name of each signal in the fluorescence excitation-emission matrix is defined by the abbreviation for its emission-light colour separated by the underscore sign from the abbreviation for its excitation light colour: blue (BF), green (GF) and far-red (FRF) fluorescence, excited by ultraviolet (UV) or red (R) light. The central wavelength of each colour is indicated in brackets. (B) Indices calculated from Multiplex® signals: blue-to-far-red emission ratio (BFRR_UV), flavonols (FLAV) and blue-to-green fluorescence emission ratio (BGFR_UV).

(A)	Excitation (nm)	Emission[a] (nm)		(B)	Ratio	Formula
	Red–orange (R) (635)	FRF_R	(735)		BFRR_UV	BF_UV/FRF_UV
	UV (375)	BF_UV	(450)		FLAV	log(FRF_R/FRF_UV)
		GF_UV	(531)		BGFR_UV[b]	BF_UV/GF_UV
		FRF_UV	(735)			

[a] Wavelengths (nm) of the excitation and emission represent the peak maxima in the used Multiplex configuration.
[b] This ratio was calculated after data acquisition.

(400 μmol CO_2 m^{-2} s^{-1}) and vapour pressure deficit (1.2 ± 0.2 kPa). The light intensity was 400 μmol m^{-2} s^{-1} with 10% blue light. For the A/Ci curves, the CO_2 was set in the following order: 400, 250, 100, 50, 400, 750, 1000, 1500, and 400 μmol m^{-2} s^{-1}. For the light–response curves, the PAR was programmed with PPFD values of 400, 200, 100, 50, 400, 700, 1000, 1500, and 400 μmol m^{-2} s^{-1}. In the case of the fluorescence kinetic measurements of dehydrated leaves, after a stable dark photorespiration, the PAR was adjusted to 400, 100, and 1000 μmol m^{-2} s^{-1}.

Thereafter, the leaf segments used for the fluorescence and gas-exchange measurements were cut for the determination of their relative water content (RWC), where

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \quad (2)$$

The turgid weight was determined after water absorption by the samples (distilled water, 24 h at 4 °C in darkness). The leaf cuticle was slightly damaged with 600-grit sandpaper wetted with a single drop of distilled water. When removing the leaves from the water, the leaf surface was gently dried with lint-free tissue. The dry weight was estimated after oven drying the samples at 50 °C for 48 h.

2.7. Data processing and statistics

To compare the behaviour of the different genotypes to the water deficit better, the raw data of the parameters recorded for the plants exposed to the water deficit were standardised against the values collected from the control plants. For this purpose, the percent modification [100 − (control/treatment * 100)] was calculated. Next, the pre-processed data were subjected to a statistical analysis using the SPSS package (PASW statistics version 18.0, SPSS Inc., Chicago, USA). The means of each treatment group, i.e., Sumo and Tulsa and Trinakria and Creso, were compared using a one-way Anova ($p \leq 0.05$). The graphs (Mean ± SE, $n \geq 6$) were drawn with SigmaPlot 8.02 (Systat Software Inc., Richmond, CA, USA).

3. Results

3.1. Morpho-physiological characterisation of the wheat cultivars

The *T. aestivum* cultivars were characterised for their photosynthetic capacity (A) under increasing CO_2 concentrations (50–1300 ppm) and light intensity (50–1500 μmol m^{-2} s^{-1} PPFD). The A/Ci curves (Fig. 1) indicate that Sumo and Tulsa have similar maximum photosynthetic rates (19 μmol CO_2 m^{-2} s^{-1} for Sumo and 21 μmol CO_2 m^{-2} s^{-1} for Tulsa). The maximum electron transport rate (ETR) was also similar for the two cultivars at approximately 90 μmol m^{-2} s^{-1} (Fig. 1A and B). Both the assimilation and the ETR increased in parallel by increasing the light intensity (Fig. 1C and D).

The visual assessments (data not shown) of the *T. durum* cultivars demonstrate that the cv. Trinakria had longer and thinner leaves than the cv. Creso. Under an appropriate water supply, Trinakria developed faster than Creso, whereas under drought the tolerant Trinakria began to wilt earlier compared to Creso. Similarly, the *T. aestivum* cultivars had pronounced differences with regard to their morphology and, in particular, their leaf characteristics. Sumo had long and thin leaves, and Tulsa had shorter and broader leaves. Tulsa developed faster than Sumo under an optimal water supply. However, under a water deficit, Sumo, which was previously classified as more tolerant to drought, began to wilt earlier than Tulsa. Similarly, Sumo had a stronger reduction in the leaf water content (Table S1). Photographs at the end of the drought

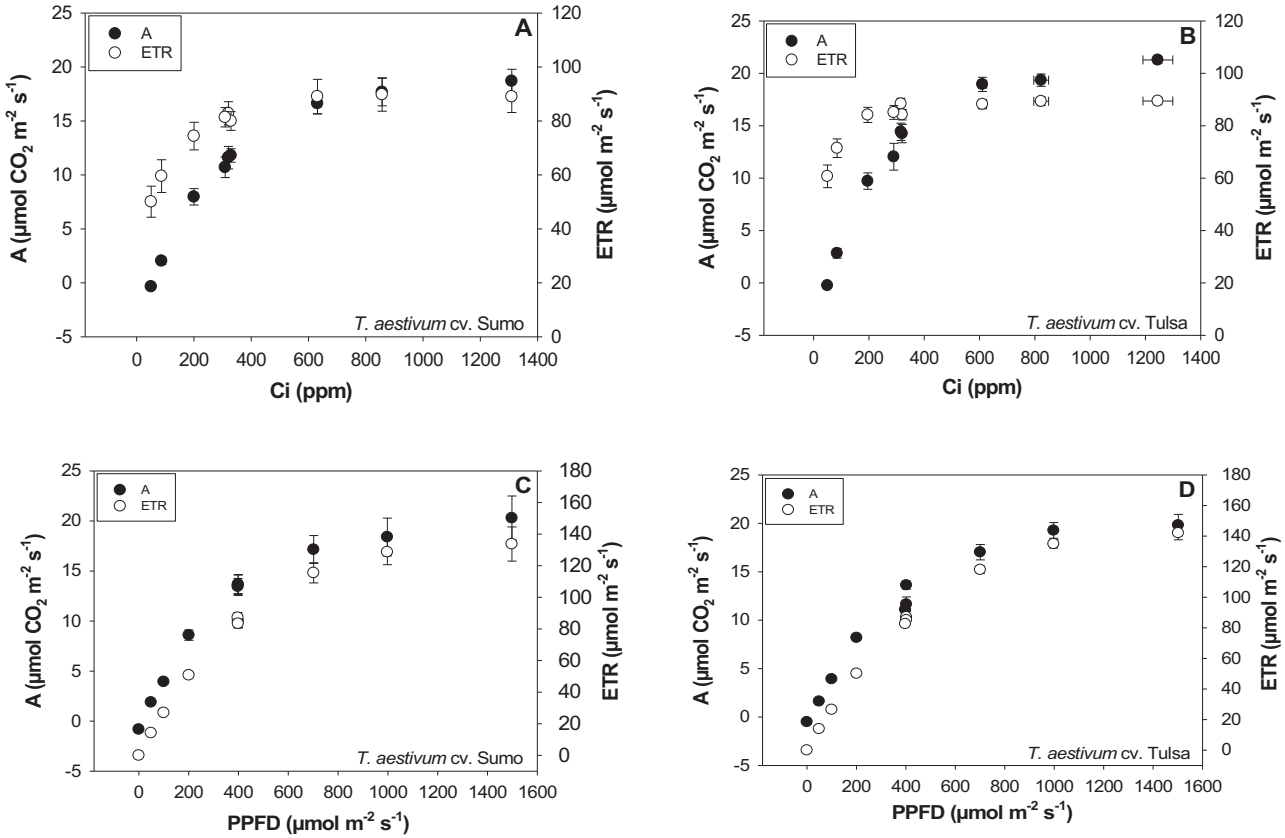

Fig. 1. Assimilation and electron transport rate of the *T. aestivum* cultivars Sumo and Tulsa, as influenced by the CO_2 concentration and light intensity (mean ± SE, $n \geq 6$).

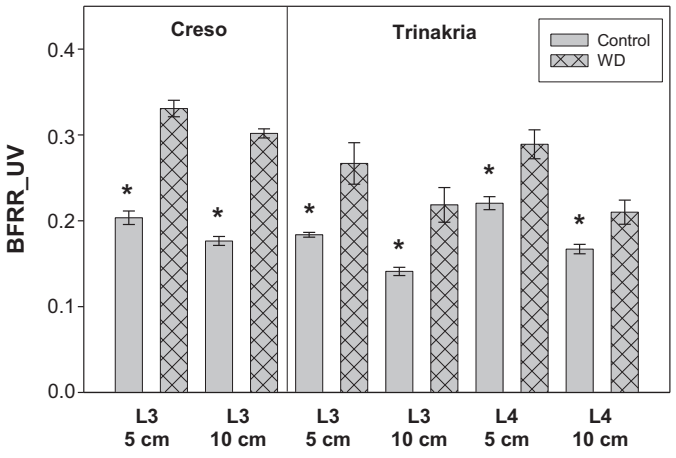

Fig. 2. Blue-to-far-red fluorescence emission ratio (BFRR_UV), as affected by the water supply and tissue age. The fluorescence was recorded from well-watered (control) and water-deficit-exposed (WD) *T. durum* plants, cultivars Trinakria and Creso. The readings were obtained on leaf 3 (L3) and leaf 4 (L4) at a distance of 5 or 10 cm from the leaf tip (greenhouse experiment; mean ± SE, $n \geq 6$). *Significant differences (Anova, $p \leq 0.05$) between the treatment groups.

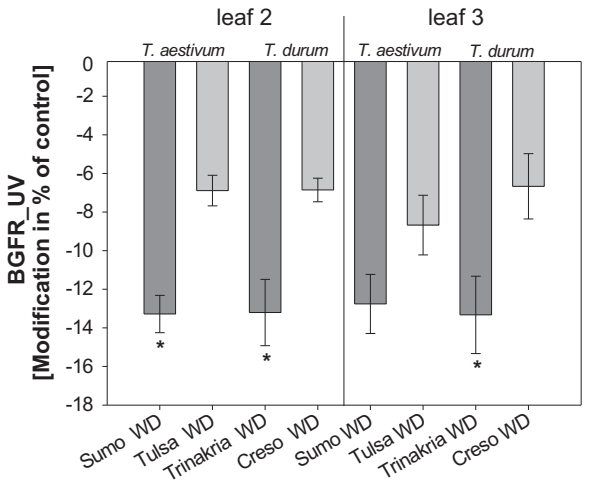

Fig. 3. Influence of leaf age and water deficit on the blue-to-green fluorescence ratio (BGFR_UV). The values (mean ± SE, $n \geq 6$) indicate the percent modification in the leaves from the water-deficit (WD) treatment compared to their respective control treatments. The measurements were obtained at 10 cm from the leaf tip at leaves 2 (L2) and 3 (L3). *Significant differences (Anova, $p \leq 0.05$) between Sumo and Tulsa or Trinakria and Creso.

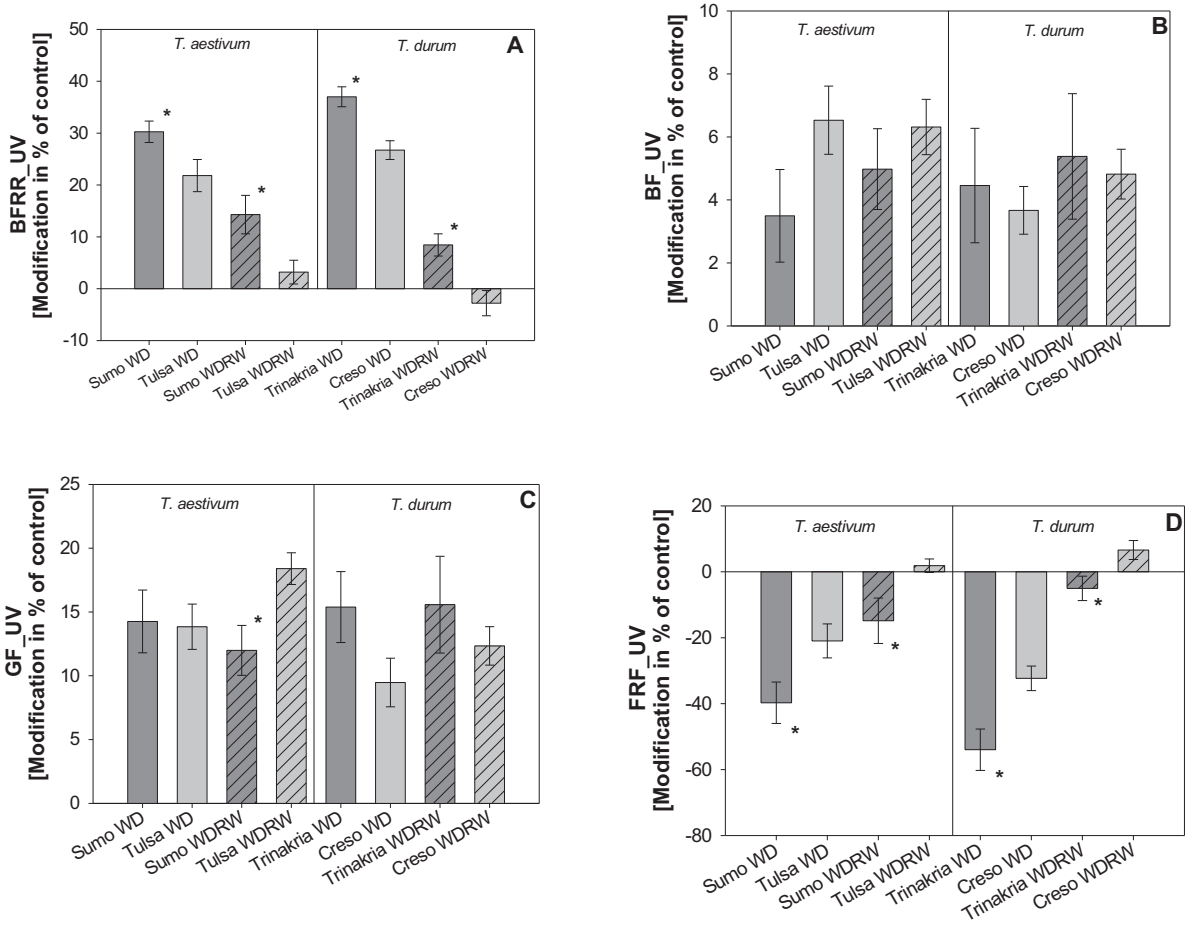

Fig. 4. Impact of water deficit on the blue, green and far-red fluorescence and the blue-to-far-red ratio. The percent modification (mean ± SE, $n = \geq 6$) of the index BFRR_UV (A) and the BF_UV (B), GF_UV (C) and FRF_UV (D) signals of leaves under water deficit (WD) and re-watered (WDRW) plants compared to the respective control plants are presented. The measurements (greenhouse plants, L3, 10 cm from the leaf tip) were performed at 23 and 25 DAS. *Significant differences (Anova, $p \leq 0.05$) between Sumo and Tulsa or Trinakria and Creso.

period show the four genotypes with wilting symptoms of different intensities (Fig. S1).

3.2. BFRR_UV and BGFR_UV: relevance of the physiological age of the tissue

In a first step, the suitability of the Multiplex® fluorescence indices to indicate drought stress was evaluated on tissues of different physiological age. For this purpose, we choose the BFRR_UV, a fluorescence emission ratio-based index, which comprises the blue and the far-red fluorescence after UV excitation. In all tested situations (i.e., cultivars Trinakria and Creso, measurements at 5 and 10 cm from the tip of L3 and L4), the BFRR_UV index significantly increased when the plants were under water deficit (Fig. 2). The LWC of L3 was approximately 80% in the control leaves and 40% and 30% for the dehydrated leaves of Trinakria and Creso, respectively.

We next analysed the drought-induced changes in the ratio between the blue and green fluorescence emission under UV excitation (BGFR_UV) in L2 and L3 of the two *T. aestivum* and two *T. durum* cultivars. The BGFR_UV was reduced in a range of 6–15% when compared to the control plants (Fig. 3). Furthermore, both cultivars classified as more tolerant (Sumo and Trinakria) had a stronger reaction than those classified as more sensitive (Tulsa and Creso). The raw signal data confirmed that the absolute intensity for both blue and green fluorescence increased, with the increase of the green fluorescence generally being more pronounced (Fig. 4B and C).

3.3. Changes in fluorescence due to drought and re-watering

In the main experiment, we evaluated the physiological response of the two *T. aestivum* and two *T. durum* genotypes to water deficit and re-watering. The four cultivars had a significantly higher BFRR_UV index at the end of the drought period, and the increase was more pronounced in both cultivars classified as drought tolerant, i.e., Sumo (30%) and Trinakria (35%). Furthermore, the BFRR_UV of all the cultivars approached the values of the control plants after re-watering (Fig. 4A).

The drought-induced increase of BFRR_UV was supported by the increase of the blue fluorescence in the range of 3–7% (Fig. 4B) and the green fluorescence in the range of 10–15% (Fig. 4C). This response was observed for all the cultivars, but, in general, there was no difference between the cultivars with regard to the magnitude of these changes. In contrast, the decrease of the UV-excited far-red fluorescence (FRF_UV) in the range of 20–60% (Fig. 4D) was the determining factor for the reduction of BFRR_UV. The far-red fluorescence approached the levels of the control plants after the re-watering of the water deficit-treated plants. Correspondingly, the percent modification was less pronounced, even though the statistical significance between the cultivars of the same genotypes remained.

In addition, we observed a decrease in the leaf width in the range of 10–40% in the drought-exposed plants (Fig. 5A), roughly resembling the pattern observed for FRF_UV (Fig. 4D). The leaf shrinkage drives the concentration of fluorescing pigments per leaf area and

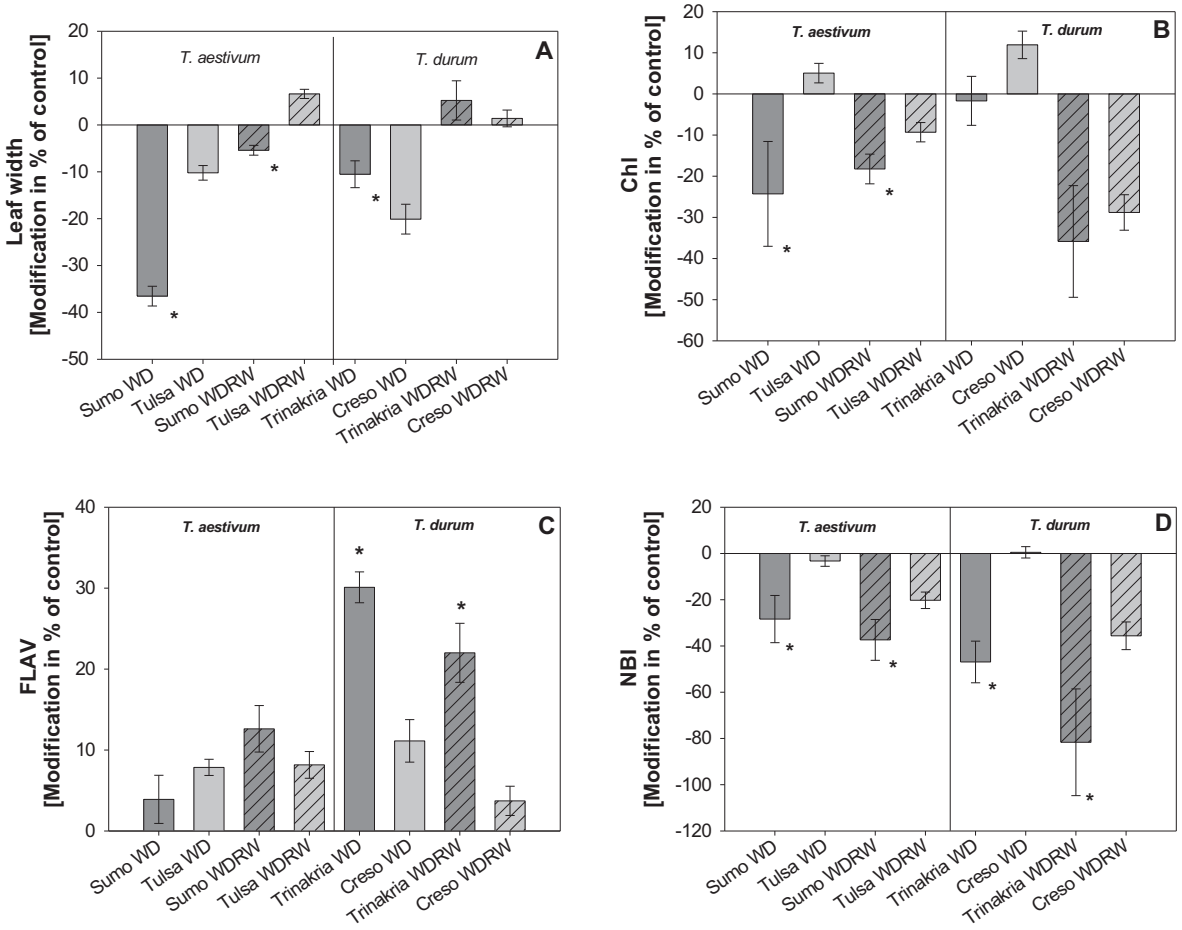

Fig. 5. Leaf width (A), chlorophyll (Chl) (B) as well as the, flavonol index (FLAV) (C) and nitrogen balance index (NBI) (D) determined using the Dualex® leaf-clip. The values (mean ± SE, $n \geq 6$) indicate the percent modification of leaves from water deficit (WD) and re-watered (WDRW) plants, as related to the respective control leaves (greenhouse experiment, leaf L3, 23 and 25 DAS; readings at 10 cm from the leaf tip). *Significant differences (Anova, $p \leq 0.05$) between Sumo and Tulsa or Trinakria and Creso.

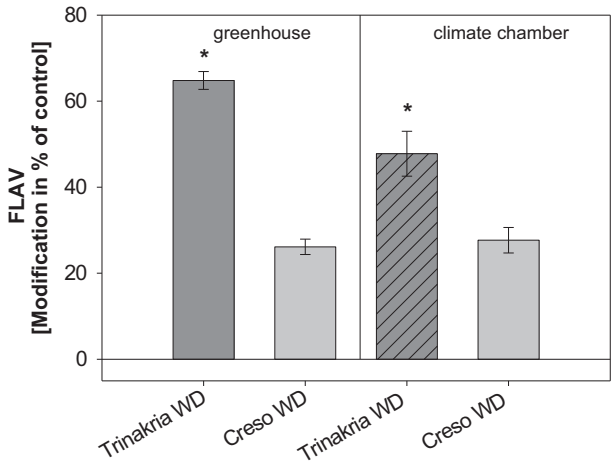

Fig. 6. The FLAV index measured with the Multiplex® sensor. The graph (mean ± SE, $n \geq 6$) illustrates the percent modification in water-deficit (WD) leaves grown under greenhouse or climate chamber conditions (L3; 10 cm from the leaf tip). *Significant differences (Anova, $p \leq 0.05$) between the cultivars.

modifies the optical properties of the leaves, which was addressed further using spot measurements with the Dualex® leaf-clip. As shown by the Chl index, the chlorophyll content was reduced in the water-deficit plants in most cases (Fig. 5B). In addition, the content of flavones in the epidermis was strongly increased, particularly in the *T. durum* cultivars, as demonstrated by the FLAV index (Fig. 5C). Lastly, the nitrogen balance index (NBI), which is related to both the chlorophyll and the nature and concentration of epidermal phenolics, was reduced, particularly in those cultivars previously classified as more drought tolerant. Furthermore, the relative changes of NBI were more pronounced in the *T. durum* cultivars, ranging from 0% in cv. Creso to −50% in the cv. Trinakria (Fig. 5D). A similar trend, i.e., a stronger NBI reduction in the cultivars considered to be drought tolerant, was observed in *T. aestivum* (Sumo, −30%; Tulsa, −5%). The stronger NBI modification in all the cultivars after re-watering is apparently a delayed effect of the drought condition and is related to the increased synthesis of pigments when the water supply was normalised.

3.4. Validation experiment: detection of stress under controlled conditions

As we found that drought induced the largest change in the FRF_UV signal (Fig. 4D), we used the Multiplex® FLAV index, which corresponds to log(FRF_R/FRF_UV), to evaluate the impact of drought on the *T. durum* cultivars under different environmental conditions. Compared to the control plants, FLAV was increased in Trinakria (65%) and Creso (25%) when cultivated in the greenhouse under a less controllable environment (i.e., temperature, % RH, and light). We observed a similar trend in the climate chamber with controlled environment, even though the increase of FLAV in Trinakria (50%) was less pronounced than in the greenhouse (Fig. 6). This difference might be related to the differences in the light quantity and quality.

4. Discussion

We evaluated the physiological response of wheat cultivars during water deficit and re-watering phases, grown under greenhouse and climate chamber conditions. Our aim was to analyse and better understand the response of drought-tolerant and drought-sensitive cultivars to water deficit during the vegetative phase, by using robust multiparametric fluorescence indices.

4.1. Fluorescence-based sensing of drought stress in wheat

Our first assumption, i.e., that the selected fluorescence-based indices might be used to sense drought-induced stress, was confirmed. We found a significant modification of the fluorescence emission ratios BGFR_UV (Fig. 3) and BFRR_UV (Figs. 2 and 4) and of the FLAV index (Fig. 6) in plants suffering from drought stress compared to well-watered control plants. These changes were observed in tissues of different physiological ages, on different leaves and for plants grown under climate chamber or greenhouse conditions. In the case of BGFR_UV (Fig. 3), the increase of this index in the stressed plants was the consequence of a disproportional increase of the fluorescence emission in the green compared to the blue spectral region.

This behaviour was also observed in the re-watering experiments (e.g., Fig. 4B and C) and is in agreement with previous reports, particularly for BFRR (Buschmann et al., 2000). The increase in BFRR_UV (Fig. 4A) was caused by a slight increase (3–6%) in BF_UV (Fig. 4B) but was mainly driven by a strong reduction (20–50%) of the FRF_UV fluorescence signal (Fig. 4D).

In general, the increase in the intensity of BF and GF in stressed plants is due, in part, to the slow decline in the quantity of pigments re-absorbing BF and GF emissions (mainly chlorophyll and carotenoids) and also in the higher epidermal concentration of compounds that absorb UV light and fluoresce in the blue and green spectral ranges (Szigeti, 2008). In particular, the cinnamic acids and other plant phenolics covalently bound to cell walls contribute to BF and GF (Lang et al., 1991; Lichtenthaler and Schweiger, 1998; Cerovic et al., 1999; Buschmann et al., 2000). It has been shown previously in drought-stressed barley leaves that BF and GF increase in the lower epidermal cell walls (15–20 μm depth) and vascular strands and that this increase is flavonoid independent (Hideg et al., 2002).

In our study, point measurements using the Dualex® device confirmed the increase in concentration of flavones (5–30%) in the epidermis (Fig. 5C) as the origin of the drought-induced changes of the BFRR index. The parallel reduction in the chlorophyll content (Fig. 5B) also contributed for a reduction of the NBI index (Fig. 5D). The changes in leaf width (Fig. 5A), which were associated with leaf shrinkage and leaf rolling, are caused by the loss of water particularly from the bulliform cells located above the midrib as a protective mechanism in monocots to avoid further water loss. This morphological adaptation could partially be responsible for the increase in the flavone content per unit surface. However, neither the FLAV index nor the Chl changes can be explained merely by the reduction of the leaf area (Fig. 5A), indicating that real changes in the secondary metabolism of the plant are occurring. In most cases, the re-watering of the plants caused the fluorescence values to approach the values of the control plants, indicating that the changes were, at least, partially reversible.

The physiological reactions of the drought-stressed plants were also demonstrated under climate chamber conditions. As exemplarily shown for the FLAV index, the genotypes Trinakria and Creso had a significantly higher FLAV index compared to the control plants, irrespective of the growth environment (Fig. 6). Furthermore, these results demonstrate the robustness of the parameter, a highly relevant attribute for stress physiology studies, which might be conducted under very different conditions.

4.2. Can the multiparametric fluorescence-based indices contribute to the selection of drought-tolerant genotypes?

Our working hypothesis was that cultivars with a similar degree of tolerance may present a similar drought-induced modification of their fluorescence patterns during the vegetative phase. Thus, we compared the behaviour of one tolerant and one sensitive

cultivar of *T. aestivum* (Sumo vs. Tulsa) and *T. durum* (Trinakria vs. Creso). In both species, the cultivars considered to be more tolerant had a stronger modification of the target fluorescence indices (e.g., BFRR_UV) compared to the cultivars previously classified as sensitive. The screening for stress-tolerant genotypes using sensors is frequently asserted as a high priority (Havaux and Lannoye, 1985; Grzesiak et al., 2003; Baker and Rosenqvist, 2004; Grzesiak et al., 2007; Hura et al., 2009; Oukarroum et al., 2009). In reality, screening experiments can be performed under very different conditions, e.g., field plots or potted plants cultivated in semi-controlled or controlled environments. Additionally, the physiological stage of the plants at the time of stress application plays a significant role. The complexity of the environment under natural systems is also a major reason why premium transgenic lines often do not outperform their controls (Saint Pierre et al., 2012). In contrast to other disciplines, the major attention in crop production is not directed to plant survival but to the yield and quality of the product (Sullivan and Eastin, 1974; Fleury et al., 2010).

To cope with water-deficit stress, plants undergo specific anatomical, morphological, physiological, biochemical and molecular adaptations to avoid dehydration by increasing their water uptake and reducing the water lost (Chaves et al., 2003). This was also observed in our study, whereby the CO_2 assimilation rate, electron transport rate and photochemical quenching decreased concomitant with an increase in non-photochemical quenching in both cultivars under increasing water deficit (Fig. S2). Nonetheless, the modification of these physiological parameters was stronger in the Sumo cultivar, which was previously classified as more tolerant, even though the light and A/Ci curves indicate a comparable photosynthetic capacity under non-stressful conditions (Fig. 1). Therefore, the tolerance to water stress of the cultivar Sumo appears to be better characterised by the fluorescence indices linked to morphological and biochemical adaptations than to the indicator parameters linked to photosynthesis and variable chlorophyll fluorescence. Indeed, in both species *T. aestivum* (Sumo vs. Tulsa) and *T. durum* (Trinakria vs. Creso), the cultivars considered to be more tolerant had a stronger modification of the target fluorescence indices (e.g., BFRR_UV) compared to the cultivars previously classified as sensitive (Figs. 3 and 4). Similar conclusions were drawn in other studies characterising the reaction of genotypes (Grzesiak et al., 2007; Hura et al., 2009). However, it is risky to extrapolate such trends to a large population without having first tested a representative number of genotypes. The re-watering of plants after the water deficit period, as we did in our trials, supports the understanding of the physiological processes leading to plant recovery. Nevertheless, it is difficult to predict how the genotypes would behave if a second stress cycle (of the same or different intensity) or a second stress type (e.g., heat, light, mineral supply, pathogens) would be imposed. At the extreme, the drought resistance in seedlings grown in pots might not correspond at all with the drought resistance during grain filling in the field (Blum, 2005). Therefore, robust indices for the rapid and non-destructive sensing of stress in agricultural crops could come from the use of multiparametric field sensors that would allow the timely initiation of the required cultural practices (cf. Tremblay et al., 2011).

As shown in present work, the blue-to-far-red fluorescence emission ratio (BFRR_UV) is a robust and reliable parameter to indicate the drought stress. From a physiological point-of-view, the leaf shrinkage and de novo synthesis of UV-absorbing chlorophyll-screening compounds were decisive in increasing this ratio. In addition, the most evaluated parameters showed a similar trend for the tolerant and susceptible cultivars of *T. aestivum* and *T. durum*, whereas the results after re-watering indicate that the specific modifications were reversible. Thus, the BFRR_UV index and the FLAV index can be considered as candidates for universal and genotype-independent parameters for drought detection in wheat. Furthermore, the selected indices provide practical tools for the management of such cultural practices as irrigation. However, these fluorescence indices should be further analysed and validated in extensive genotype screening programmes and field experiments.

Conflicts of interest

ZGC declares a double link to the FORCE-A company:as one of the co-authors of the Dualex® and Multiplex® patents that the company exploits and as a part-time consultant to the company. Other authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2013.01.003.

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