

Article

Analysis of Physiological Status in Response to Water Deficit of Spelt (*Triticum aestivum* ssp. *spelta*) Cultivars in Reference to Common Wheat (*Triticum aestivum* ssp. *vulgare*)

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Abstract: Climate change, including decreasing rainfall, makes cultivating cereals more difficult. Drought stress reduces plant growth and most all yields. On the other hand, consumers' interest in ancient wheat varieties, including spelt, is growing. The aim of this work is to compare the response to drought stress between spelt (*Triticum aestivum* ssp. *spelta*) and common wheat (*Triticum aestivum* ssp. *vulgare*). Six cultivars of spelt from different European countries and common wheat 'Bogatka' as a reference were chosen for research. The photosynthesis process, chlorophyll fluorescence, relative water content, and the content of free proline and anthocyanins in well-watered and drought-stressed plants were measured. It was shown that the spelt cultivars 'Franckenkorn' and 'Badengold' were much more resistant to water deficit than other cultivars and even common wheat. A slight reduction of CO₂ assimilation (by 27%) and no reduction of transpiration rate, with simultaneous intensive proline (eighteen times fold increase) and anthocyanins accumulation (increase by 222%) along with a slight increase in lipid peroxidation level (1.9%) revealed in 'Franckenkorn' prove that this cultivar can cope with drought and can be effectively cultivated in areas with limited water abundance.

Keywords: drought; chlorophyll fluorescence; MDA; photosynthesis; proline; RWC; spelt cultivars



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1. Introduction

Low water availability causes soil drought, one of the most serious worldwide problems that largely limits plant growth and development and leads to enormous crop losses. Global climate change and the resultant water scarcity are significant threats to plant productivity on an increasing area of the Earth [1–4]. Consequently, more attention has been paid recently to identifying plant responses related to drought stress resistance mechanisms. These mechanisms include gas exchange, chlorophyll fluorescence, relative water content, lipid peroxidation index, proline, and anthocyanins accumulation, and facilitate the development of different genetic approaches to improve plant drought resistance and prevention of yield loss in economically important wheat. In contrast to common wheat, only a few studies assessed the spelt wheat's resistance to water shortages [5–8]. The effect of drought stress on the physiological properties and the course of the photosynthesis process in spelt wheat have not been fully characterized and explained. One of the negative effects of drought is the dehydration of cells which causes a drop in turgor pressure, restriction of cell elongation, stomatal closure, and the reduction of photosynthetic activity leading to growth

inhibition and a reduction in the accumulation of biomass and yield [9–11]. Stomatal closure is beneficial for plant survival under drought since it reduces water loss from leaves. However, at the same time, it causes a decrease in the CO₂ assimilation rate, which may lead to non-stomatal down-regulation of photosynthetic metabolism. Plants thus absorb more light than can be used in photosynthetic carbon fixation. This triggers reactive oxygen species (ROS) generation and damage of cell membranes due to lipid peroxidation, which leads to further inhibition of photosynthesis [12,13].

Cellular adjustments for drought tolerance include an increased concentration of protective compounds (sugar osmoprotectants, amino acids, flavonoids), proteins that condition the proper structure of cell membranes, as well as the activation of enzymatic and non-enzymatic antioxidant systems [14–18]. Proline accumulation is a common physiological response to water deficit or increased osmotic pressure. It improves osmotic regulation and cell water retention, leading to a reduction in cell dehydration and stabilization of the structure of proteins and cell membranes. It acts as a protective agent for many enzymes and an antioxidant and free radical scavenger [19]. Of the various mechanisms enabling plants to cope with water stress, the most common is the accumulation of secondary metabolites like flavonoids or anthocyanins [20,21]. Apart from playing varied roles in plant growth and development, they are also responsible for the resistance to biotic and abiotic stresses [20].

Different wheat and spelt cultivars may develop different stress resistance strategies (stress avoidance and/or stress tolerance); hence, to preserve growth and productivity, there is a need to broaden the knowledge about the mechanisms which enable plants to rapidly cope with drought stress [22]. Identification of cultivars with increased stress resistance is possible by observation of the genetic variations of several spelt cultivars in response to drought stress and investigation of the differences in possible mechanisms involved in this resistance with reference to common wheat. In recent years, measurements of photosynthesis and chlorophyll fluorescence have been very popular as they enable the identification of the physiological activity of plants under biotic and abiotic stress conditions [23,24]. Detailed monitoring of plant physiology, the basis of photosynthesis regulation under imposed conditions of water scarcity, is crucial for the estimation of resistance to drought.

Spelt wheat has been attracting renewed interest worldwide due to the growing demand for traditional and organic products and so-called functional food [25,26]. Being richer in nutrients in comparison to other cereals, spelt products with no other cereal admixtures display a pro-health effect [27]. Organic and sustainable agriculture drives the demand for cereal species and cultivars, which can cope with drought and provide a stable yield under these adverse environmental conditions without requiring high-input cultivation [28]. At the same time, spelt wheat is suitable for cultivation in soils that are poorer in minerals. In recent years, special attention has also been paid to the need to preserve genetic biodiversity in agro-ecosystems [29].

This study aimed to compare the suitability of selected cultivars of spelt wheat for cultivation under drought stress in relation to common wheat. The research hypothesis assumed that some of the examined cultivars of spelt wheat might show greater drought stress resistance. Six spelt wheat cultivars were collected. Variations in drought responses were evaluated based on the relative water content in leaves (RWC), gas exchange and chlorophyll fluorescence parameters, lipid peroxidation level, proline, and anthocyanins content. Common wheat cultivar was used as a reference. Plant physiological activity under well-watered and drought conditions was determined, and the drought resistance index (DRI) was calculated. The differences in the drought resistance of the examined wheat cultivars were analyzed concerning the destructive (lipid peroxidation) and protective (proline and anthocyanins accumulation) changes at the metabolic level. The relationships between physiological parameters were further comparatively dissected and discussed. The results provided some insights into the understanding of the physiological mechanisms

of spelt drought tolerance. They indicated the most useful cultivar comparable to common wheat for growing in difficult water stress conditions.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The experiments were performed in three annual cycles (2013, 2014, 2015). The following six winter spelt wheat: 'Franckenkorn', 'Oberkulmer Rotkorn', 'Badengold', 'Divimar', 'Schwabenkorn' and 'Ostro' cultivars were analyzed with reference to one common wheat 'Bogatka' cv. The seed material (spikelets) was purchased from breeding companies. The pot experiment was performed in the experimental field belonging to the Department of Agronomy of the Poznań University of Life Sciences (Poznań, Poland). Plastic polyethylene pots (6 L) were filled with 4 kg of a universal substrate (pH 5.5) (Kronen, Lasland Sp. z o.o. Cerekwica). In each pot, 5 spikelets of cultivars of spelt wheat and grain of common wheat were sown in four replicates in each independent experiment. All these cultivations of plants were conducted according to a randomized complete block design and kept in the same growth conditions. The sowing date was set in accordance with the recommendations for the Greater Poland Voivodeship for the first five days of October. The three most aligned plants (healthy cuttings in pots of nearly the same size) were left in each pot after seed germination (14 days). On the next day, the first fertilization using Florovit (Grupa Inco S.A., Góra Kalwaria, Poland) liquid fertilizer (0.5 mL/100 mL H₂O/pot) and ammonium nitrate (1 g /100 mL H₂O/pot) was performed. Fertilization was repeated twice at 14-day intervals. Pots with plants were kept outdoor (natural conditions) in the winter period, but in spring, they were moved to a greenhouse of the Department of Agronomy (60% to 80% relative humidity, 20 to 25 °C, 16 h day/8 h night). To assess the potential drought tolerance of spelt varieties, plants were subjected to watering conditions (control) and drought condition (soil water deficit) by withholding water in the soil for 10 days. Plants were grown under natural sunlight supplemented with sodium lamps light (HPS) with a power of 400 W and PPFD 700 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}$ (Elektro-Valo Oy Netafim, Avi:13473, Uusikaupunki, Finland). Soil moisture was monitored daily with a probe (ThetaProbe, Eijkelkamp, The Netherlands). Drought stress was imposed by stopping the watering at the flowering phase of the plants (BBCH 69). After 10 days of drought, the soil moisture content reached 6–8% *v/v*, and was unavailable to the plants. Leaves lost their vigor and started to wither. The control plants of all cultivars were provided with an optimal soil moisture content of 20 to 22% *v/v*. The physiological state of the plants was determined in both control and the drought-stressed plants at the end of the drought period. After completing the measurements, all the leaves were cut off and frozen in liquid nitrogen for further laboratory analyses, including the determination of free proline, anthocyanins, and lipid peroxidation levels. Relative water status (RWC) was determined immediately after harvest. The experiments used four independent biological replicates. Each replicate was a sample of plant material derived from a different pot. The physiological measurements (gas exchange, chlorophyll fluorescence, chlorophyll content index) were performed in three identical annual cycles (2013, 2014, 2015), while laboratory measurements (RWC, lipid peroxidation, proline, and anthocyanin accumulation) were performed in two years (2013, 2014).

2.2. Gas Exchange Parameters

Before the measurements were started, the plants were dark-acclimated for 9 h. The measurement was carried out in a phytotron at a constant air temperature of 25 °C and an ambient humidity of 70% \pm 5%. The physiological state of the plants and their photosynthetic activity were determined using the LCpro-SD (ADC BioScientific Ltd., Hoddesdon, UK) and the following parameters: A-CO₂ assimilation level ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), E-transpiration ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Gs-stomatal conductance ($\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Ci-intercellular CO₂ concentration (vpm). The measurement sequence was the same, and the stressed and control plants were alternately measured for each analyzed cultivar. For each pot, the two

youngest fully developed leaves from two plants were analyzed. The measurement was performed on four pots (a total of 8 measurements per combination). The concentration of CO₂ supplied to the measuring chamber (reference CO₂) was kept at 360 vpm. The airflow to the measuring chamber (u) was maintained at 200 µmol/s. The concentration of H₂O (reference H₂O) was set to ambient, i.e., the actual concentration in the environment. The intensity of the light emitted in the measuring chamber (PPFD-photosynthetic photon flux density) by the red and blue LEDs (in the proportion of 10:1) of the spectrum was set to 400 µmol·m⁻²·s⁻¹ (LCP Narrow Lamp, ADC BioScientific Ltd., Hoddesdon, UK).

2.3. Chlorophyll Fluorescence

Chlorophyll fluorescence was measured using a Fluorometer OS5p (Optosciences Inc., Hudson, NH, USA) with a PAR clip that measures photosynthetically active radiation (PAR), leaf temperature, and calculates the electron transport rate (ETR) parameter during the measurement. The kinetic protocol was selected, and measurements on plants after dark adaptation and under light were taken. The following parameters were measured: F₀-minimum fluorescence, F_m-maximum fluorescence, F_v/F_m-maximum PSII quantum yield in the dark-acclimated state, Y (PSII)-PSII quantum yield in the light-acclimated state, ETR-Electron Transport Rate. Chlorophyll fluorescence measurement was performed in the same, youngest, fully developed two leaves from every pot selected for the gas exchange measurement (2.2.). The Modulation Source was set to red with an intensity of 22 in the possible range from 1 to 32, where 17 is for an approximate value of 0.1 µmols. The optimal setting is the highest possible intensity that does not induce variable fluorescence. The Saturation Flash was set to an intensity of 30 in the possible range from 1 to 32, where 32 is an approximate value of 8550 µmols. The measurement cycle was set to two saturation pulses 180 s apart. The number of flashes and the duration of measurements were selected based on the time each plant needed to completely silence the fluorescence. At the beginning of the measurement, the weak modulated source is started, and the parameter F₀ is measured. The next step is to turn on a single saturation pulse and measure the F_m value. Then the reaction centers of the PSII are closed. When the fluorescence signal returns to the minimum value, actinic light is turned on (about 400 µmol·m⁻²·s⁻¹), which triggers biochemical reactions and allows the calculation of Y (PSII) and ETR parameters. The equation of ETR is $ETR = (Y (II) (0.84) (0.5) PAR$.

2.4. Chlorophyll Content Index

Chlorophyll Content Index (CCI) was measured using a CCM-200 plus (Optosciences Inc., Hudson, NH, USA). The same, youngest, fully developed two leaves were selected for the CCI measurement, as for chlorophyll fluorescence and gas exchange measurement. The CCM-200 Plus chlorophyll meter is used to determine the relative concentration of chlorophyll by measuring optical absorbance in two wavebands: 653 nm (Chlorophyll) and 931 nm (Near Infra-Red).

2.5. Relative Water Content (RWC)

Plant water status was determined by measuring leaf RWC of control and drought-stressed plants. Two-centimeter leaf slices, freshly collected from control and drought-stressed plants, were weighed three times in weighing dishes. The first weighing was carried out immediately after collecting the plant material (fresh mass; f.m.), the second after soaking the plant material for 4 h in distilled water (f.m. in full turgor), and the third weighing was carried out after drying the plant material for 4 h in 70° C (dry mass; d.m.). RWC was calculated using the formula:

$$RWC [\%] = \frac{f.m. - d.m.}{f.m. \text{ in full turgor} - d.m.} \quad (1)$$

2.6. Lipid Peroxidation Index (MDA)

The level of lipid peroxidation in the leaves of control and drought-stressed plants was determined by quantifying the malondialdehyde (MDA) content using the thiobarbituric acid assay (TBA test) according to Heath and Packer [30]. Plant material (200 mg fresh matter) was homogenized with 4 mL of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at $5000 \times g$ for 15 min. and used to assess MDA content. The mixture of 2 mL of 20% TCA containing 0.5% TBA was added to 0.5 mL of the supernatant, heated to 95°C for 30 min, then cooled in an ice bath and centrifuged at $10,000 \times g$ for 10 min. The absorbance of the supernatant was measured at wavelengths $\lambda = 532\text{ nm}$ and $\lambda = 600\text{ nm}$. The value of nonspecific absorption at 600 was subtracted from the reading at 532 nm. MDA concentration was calculated using the molar absorption coefficient of the MDA-TBA complex equal to $155\text{ L}/\text{mmol} \times \text{cm}$ and expressed in $\mu\text{mol}\cdot\text{g}^{-1}\text{ d.m.}$

2.7. Free Proline Analysis

The free proline content in the leaves of control and drought-stressed plants was determined by the method of Bates et al. [31]. Plant material (200 mg fresh matter) was homogenized with 4 mL of 5% TCA (*v/v*). The homogenate was centrifuged at $5000 \times g$ for 15 min. The supernatant was used for proline determination by measuring the quantity of the colored reaction product of proline with ninhydric acid. The absorbance of the produced complex was measured at a wavelength of 515 nm. The amount of free proline was calculated based on the calibration curve and expressed in $\text{mg}\cdot\text{g}^{-1}\text{ d.m.}^{-1}$.

2.8. Anthocyanin Analyses

The content of anthocyanin pigments in the leaves of control and drought-stressed plants was determined by the method of Wang et al. [32]. Plant material (200 mg fresh matter) was homogenized with 3 mL of 0.5 N HCl and centrifuged at $6000 \times g$ for 10 min. The absorbance of the supernatant was measured at 530 nm. The level of anthocyanins in leaf tissue was calculated using a calibration curve of cyanine chloride and was expressed as $\mu\text{g}\cdot\text{g}^{-1}\text{ d.m.}$

2.9. Estimation of Drought Resistance Index (DRI)

To assess the drought tolerance of the studied cultivars, the resistance index (RI) was calculated based on the equation proposed by Balboa et al. (2020) [33]. For this purpose, the plant physiological activity (PPA) in drought-stressed and control plants was estimated, taking into account the following input data: RWC (%), A (unit value), Y (PSII) (unitless) value below 1), Fv/Fm (unitless) value below 1) that have the same weight in the equation. A 0 to 1 scale was used in the equation for each parameter; therefore, the RWC was divided by 100 (to eliminate the percentages) and A_d (the A value for the drought) by the A_c (the A value for the control). The physiological activity of drought-stressed plants was calculated according to the following equation:

$$\text{PPA}_{\text{wd}} = [(0.25 \times \text{RWC}_{\text{wd}}/100) + (0.25 \times A_{\text{wd}}/A_c) + (0.25 \times Y(\text{PSII})_{\text{wd}}) + (0.25 \times \text{Fv}/\text{Fm}_{\text{wd}})] \times 100 \quad (2)$$

For the conditions of control, the above equation takes the form:

$$\text{PPA}_c = [(0.25 \times \text{RWC}_c/100) + (0.25 \times 1) + (0.25 \times Y(\text{PSII})_c) + (0.25 \times (\text{Fv}/\text{Fm})_c)] \times 100 \quad (3)$$

Drought resistance index (DRI) was calculated according to the following equation:

$$\text{DRI} = \text{PPA}_{\text{wd}}/\text{PPA}_c \quad (4)$$

where PPA_c = Plant Physiological Activity in control condition. PPA_{wd} = Plant Physiological Activity in Water Deficit conditions. Therefore, DRI values close to 0 indicate that the cultivar had a lower resistance to drought, while cultivars with RI values close to 1 indicate a higher resistance to drought stress conditions.

2.10. Statistical Analysis

The effect of two factors (drought stress and cultivars) on the physiological state of plants was examined using two-way ANOVA with four replicates per cultivar accordingly to the method described previously [34]. The principal components analysis was carried out using the R software (v 4.0.5, The R Foundation, Vienna, Austria), and biplots were used to illustrate the obtained results [35]. The relationship between examined parameters for each variety was determined using the Pearson correlation coefficient. Server heatmapper was used to design cluster heat maps. Blue and yellow indicate negative and positive correlations, respectively. The hierarchical clustering heat map method was used for data analyses. Distance measure using Euclidean and clustering algorithm using Ward's linkage [36].

3. Results

3.1. Physiological State of Plants

3.1.1. Gas Exchange Parameters

Examined wheat cultivars differed in the level of gas exchange parameters in control conditions (Table 1). Drought stress caused a decrease in all these parameters, but to an extent that was different depending on the cultivar. The greatest inhibition of CO₂ assimilation rate was observed in 'Schwabenkorn' (decrease of 7.46 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, i.e., 98%), whereas the weakest inhibition in 'Franckenkorn' (decrease of 1.87 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or 27%). 'Schwabenkorn' was also characterized by the highest reduction in transpiration rate (decrease of 1.47 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, i.e., 91%); the smallest decrease was observed in the 'Badengold' cultivar (decrease of 0.1 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, i.e., 7%). Whereas, in spelt wheat, the 'Franckenkorn' cultivar transpiration rate did not change under drought stress conditions. The greatest decrease of stomatal conductance (by 0.09 $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was recorded in 'Badengold' (82%) and 'Schwabenkorn' (100%), whereas the smallest (0.03 $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in 'Franckenkorn' (43%) and 'Divimar' (75%) cultivars. The intercellular CO₂ concentration dropped the least, by 22.6 vpm (12%) in 'Franckenkorn', in turn the most, by 211.8 vpm (57%), in 'Oberkulmer Rotkorn' cultivar.

Table 1. Effect of wheat cultivars and drought stress on photosynthesis parameters.

Cultivars	A ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)		E ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)		Gs ($\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)		Ci (vpm)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Franckenkorn	6.89 ^d	5.02 ^e	1.18 ^e	1.18 ^e	0.07 ^e	0.04 ^f	182.6 ^f	160.0 ^g
Oberkulmer Rotkorn	4.29 ^f	0.34 ^j	0.79 ^{fg}	0.06 ^l	0.04 ^f	0.00 ⁱ	373.9 ^a	162.1 ^g
Badengold	9.67 ^a	2.95 ^g	1.33 ^c	1.23 ^d	0.11 ^a	0.02 ^g	201.1 ^e	169.2 ^g
Ostro	7.63 ^c	2.69 ^{gh}	1.49 ^b	0.51 ^h	0.08 ^d	0.02 ^g	291.7 ^c	143.9 ^g
Divimar	4.24 ^f	1.67 ⁱ	0.84 ^f	0.33 ^j	0.04 ^f	0.01 ^h	298.7 ^c	136.3 ^{gh}
Schwabenkorn	7.62 ^c	0.16 ^j	1.61 ^a	0.14 ^k	0.09 ^c	0.00 ⁱ	321.3 ^b	193.1 ^f
Bogatka	8.26 ^b	2.50 ^h	1.50 ^b	0.39 ⁱ	0.08 ^d	0.01 ^h	270.1 ^d	137.3 ^{gh}
LSD $\alpha_{0.05}$	0.533		0.065		0.005		29.05	

A-CO₂ assimilation level, E-transpiration rate, Gs-stomatal conductance, Ci-intercellular CO₂ concentration. The results were compiled based on 12 repetitions (4 independent biological repetitions yearly). Different letters a-l indicate statistically different mean values ($\alpha = 0.05$).

3.1.2. Chlorophyll Fluorescence and Chlorophyll Content Index

Significant loss of leaf chlorophyll in drought-stressed plants was observed in all the examined cultivars (Figure 1A). The highest decrease was observed in the 'Franckenkorn' and 'Oberkulmer Rotkorn' (66% and 48%, respectively).

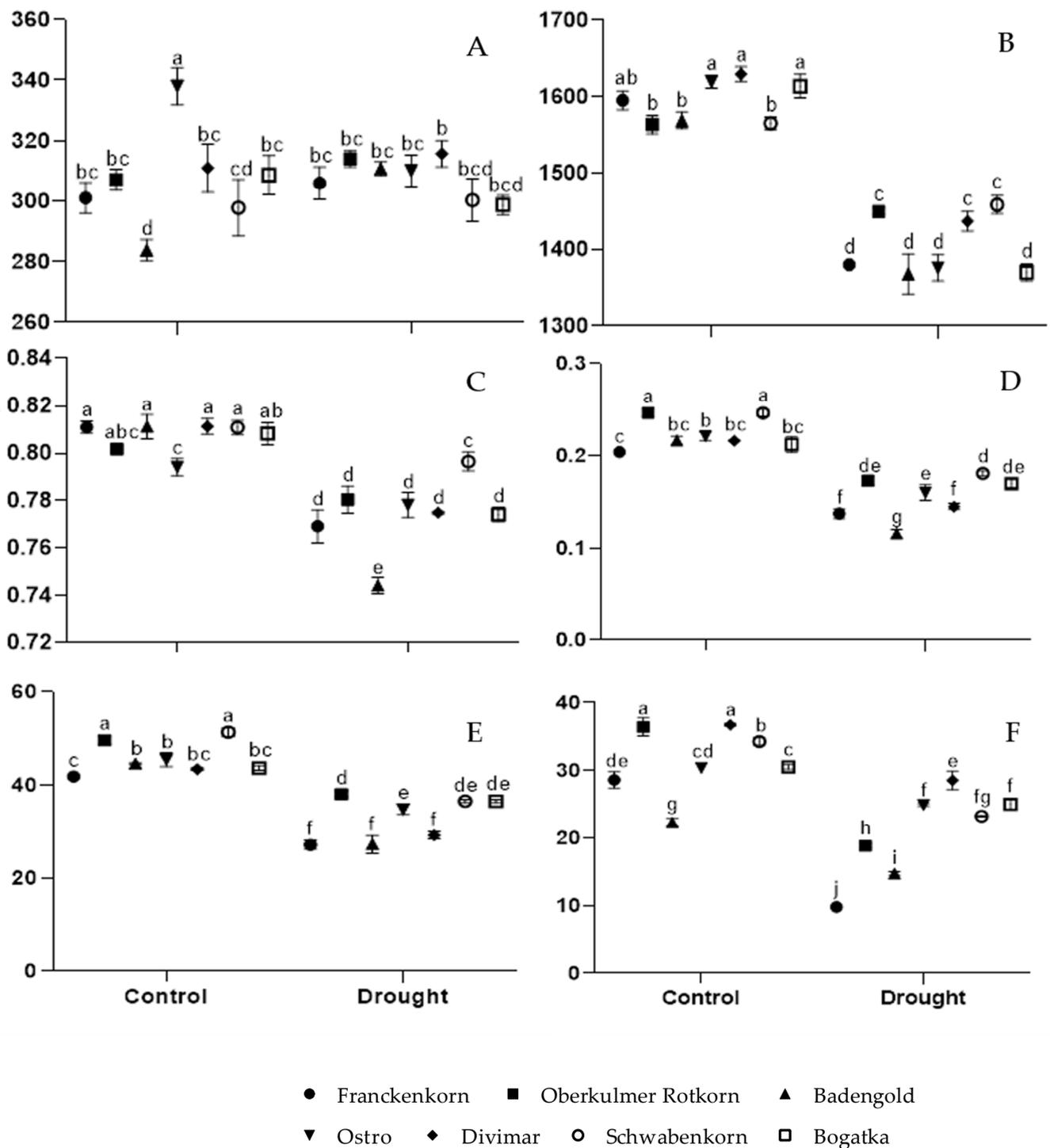


Figure 1. Parameters of chlorophyll fluorescence after dark adaptation: (A)—minimum fluorescence (F0), (B)—maximum fluorescence (Fm), (C)—maximum PSII quantum yield in the dark-acclimated state (Fv/Fm) on the light: (D)—PSII quantum yield in the light-acclimated state (Y (PSII)), (E)—Electron Transport Rate (ETR) and (F)—Chlorophyll Content Index (CCI) [non-nominated units]. The results were compiled based on 12 repetitions (4 independent biological repetitions yearly). Letters a–j indicate statistically different mean values ($\alpha = 0.05$).

Drought caused damage to PSII, resulting in significant decreases in the PSII quantum yield in the light-acclimated state-Y (PSII), as well as the rates of electron transport (ETR) in all the analyzed cultivars (Figure 1B,C). The decreases in the Y (PSII) parameter between the

control plants and the drought-stressed ones ranged from 0.04 units (19%) for the common wheat 'Bogatka' to 0.1 units (45%) for the 'Badengold' spelt wheat. Similarly, the decrease in the ETR due to drought stress was the smallest in 'Bogatka' (7.2 units, i.e., 16%), while the highest in 'Badengold' (17.3 units, i.e., 39%).

Drought stress resulted in a significant decrease (28 units, i.e., 8%) in the minimum fluorescence in 'Ostro' (Figure 1D). Otherwise, 'Badengold' showed a significant increase in the value of this parameter (27 units, i.e., 10%). No significant changes in the F0 value were observed in the remaining cultivars. Maximum fluorescence showed a significant decrease in examined cultivars which ranged from 106.8 units (7%) in 'Schwabekorn' to 243.8 units (15%) in 'Ostro' (Figure 1E). In all the examined cultivars, drought stress also caused a decrease in maximum PSII quantum yield in the dark-acclimated state (Fv/Fm) (Figure 1F). The smallest decrease of Fv/Fm was observed in 'Ostro' and 'Schwabekorn' and amounted to 0.01 units (1%). In the remaining cultivars, the decrease of photochemical efficiency of PSII was in the range of 0.02 units (2.5%) in 'Oberkulmer Rotkorn' to 0.07 units (9%) in 'Badengold'.

3.2. Relative Water Content (RWC)

RWC in leaves of control plants ranged from 88.3% to 93.1% in 'Oberkulmer Rotkorn' and 'Bogatka', respectively (Table 2). Drought resulted in a significant reduction of RWC in all the examined cultivars. In drought-stressed plants, the lowest RWC level (54.1%) was observed in the leaves of 'Schwabekorn' and the highest (85.9%) in 'Bogatka', indicating severe to mild leaf dehydration, respectively. Severe water deficit was also shown in the leaves of 'Ostro' and 'Divimar' (RWC 64.1%), slightly milder in 'Franckenkorn', 'Oberkulmer Rotkorn' and 'Badengold' (RWC 72.1 to 71%).

Table 2. Effect of wheat cultivars and drought stress on the Relative Water Content (RWC) and Lipid Peroxidation Index (MDA).

Cultivars	RWC (%)		MDA ($\mu\text{mol}\cdot\text{g}^{-1}\text{ d.m.}$)	
	Control	Drought	Control	Drought
Franckenkorn	89.2 ^b	72.1 ^d	0.26 ^c	0.31 ^c
Oberkulmer Rotkorn	88.3 ^b	71.3 ^d	0.25 ^c	0.40 ^b
Badengold	87.5 ^b	71.0 ^d	0.29 ^c	0.39 ^b
Ostro	88.4 ^b	64.1 ^e	0.27 ^c	0.33 ^b
Divimar	91.5 ^{ab}	64.1 ^e	0.25 ^c	0.50 ^a
Schwabekorn	91.2 ^{ab}	54.1 ^f	0.29 ^c	0.44 ^a
Bogatka	93.1 ^a	85.9 ^c	0.31 ^c	0.42 ^{ab}
LSD $\alpha_{0.05}$	2.99		0.09	

Different letters a–f indicate statistically different mean values ($\alpha = 0.05$). The results were compiled based on 8 repetitions (4 independent biological repetitions yearly).

3.3. Lipid Peroxidation Index (MDA)

The level of lipid peroxidation in leaves of control plants was rather similar in all examined cultivars and ranged from 0.25 to 0.31 $\mu\text{mol}\cdot\text{g}^{-1}\text{ d.m.}$ (Table 2). A statistically significant increase in lipid peroxidation was shown in all examined cultivars except for 'Franckenkorn'. The highest MDA increase (by 0.25 $\mu\text{mol}\cdot\text{g}^{-1}\text{ d.m.}$ i.e., 100%) was shown in 'Divimar', and the lowest (by 0.06 $\mu\text{mol}\cdot\text{g}^{-1}\text{ d.m.}$ i.e., 22%) in 'Ostro'.

3.4. Free Proline Accumulation

Free proline levels in leaves of control plants did not differ significantly in examined cultivars and ranged from 0.4 $\text{mg}\cdot\text{g}^{-1}\text{ d.m.}$ in 'Oberkulmer Rotkorn' and 'Schwabekorn' to 1.0 $\text{mg}\cdot\text{g}^{-1}\text{ d.m.}$ in 'Badengold' (Table 3). Drought stress caused significant increases

in the free proline content in all the cultivars. The greatest increase was found in leaves of 'Badengold' (by $18.7 \text{ mg}\cdot\text{g}^{-1} \text{ d.m.}$, which means an eighteen-fold increase), whereas a milder increase was observed in 'Bogatka' (by $4.8 \text{ mg}\cdot\text{g}^{-1} \text{ d.m.}$, which means more than sixfold increase). Among spelt wheat, 'Franckenkorn' and 'Schwabenkorn' cultivars were characterized by a significantly higher increase in proline content under water stress conditions which amounted to 11.2 and $13.4 \text{ mg}\cdot\text{g}^{-1} \text{ d.m.}$ (meaning an 18 and 33 times fold increase, respectively).

Table 3. Effect of wheat cultivars and drought stress on free proline and anthocyanins accumulation.

Cultivars	Proline ($\text{mg}\cdot\text{g}^{-1} \text{ d.m.}$)		Anthocyanins ($\mu\text{g}\cdot\text{g}^{-1} \text{ d.m.}$)	
	Control	Drought	Control	Drought
Franckenkorn	0.6 ^e	11.8 ^b	159.5 ^e	514.1 ^a
Oberkulmer Rotkorn	0.4 ^e	9.8 ^c	119.4 ^f	547.6 ^a
Badengold	1.0 ^e	19.7 ^a	199.3 ^e	302.1 ^d
Ostro	0.4 ^e	6.7 ^c	141.9 ^{ef}	426.8 ^b
Divimar	0.7 ^e	9.7 ^c	112.2 ^f	513.2 ^a
Schwabenkorn	0.4 ^e	13.8 ^b	142.9 ^{ef}	426.4 ^b
Bogatka	0.7 ^e	5.5 ^d	191.4 ^e	378.2 ^c
LSD $\alpha_{0.05}$	3.97		79.88	

Different letters a–f indicate statistically different mean values ($\alpha = 0.05$). The results were compiled based on 8 repetitions (4 independent biological repetitions yearly).

3.5. Anthocyanins Accumulation

The levels of anthocyanins in the leaves of control plants (Table 3) were the highest in 'Bandegold' and 'Bogatka' (199.3 and $191.4 \mu\text{g}\cdot\text{g}^{-1} \text{ d.m.}$, respectively), the lowest in 'Divimar' and 'Oberkulmer Rotkorn' (112.2 and $119.4 \mu\text{g}\cdot\text{g}^{-1} \text{ d.m.}$, respectively). Drought stress had a significant effect, yet the magnitude of increments in the content of these pigments was cultivar-dependent (Table 3). The highest increase was shown in 'Oberkulmer Rotkorn' and 'Divimar' (by 428.2 and $401.0 \mu\text{g}\cdot\text{g}^{-1} \text{ d.m.}$, more than threefold increase respectively), the lowest-in 'Badengold' (by $102.8 \mu\text{g}\cdot\text{g}^{-1} \text{ d.m.}$, i.e., 51%).

3.6. Physiological Plant Activity (PPA) and Drought Resistance Index (DRI)

Estimating the DRI allowed for categorizing varieties according to the degree of resistance to drought (Table 4). The physiological activity of the cultivars under control conditions (PPA_c) was similar and ranged from 72% (Ostro) to 74% (Divimar, Schwabenkorn, Bogatka). The drought contributed to the reduction of physiological activity (PPA_{wd}). The lowest PPA_{wd} index was observed for 'Schwabenkorn' (38.5%) and the highest for 'Franckenkorn' (58.9%). This variety was also characterized by the highest DRI (0.811). Slightly lower DRI was found in 'Bogatka' (0.713) and the lowest in 'Schwabenkorn' (0.518).

Table 4. Effect of wheat varieties and drought stress on physiological plant activity and drought resistance index (%).

Cultivars	PPA _c (%)	PPA _{wd} (%)	DRI (PPA _{wd} /PPA _c)
Franckenkorn	73	58.9	0.811
Oberkulmer Rotkorn	73	43.6	0.595
Badengold	73	46.1	0.636
Ostro	72	48.3	0.666
Divimar	74	48.9	0.664
Schwabenkorn	74	38.5	0.518
Bogatka	74	52.6	0.713

3.7. Principal Component Analysis

Principal component analysis (PCA) for the effect of drought stress on the examined wheat cultivars is presented in Figure 2. Biplots were constructed based on data from laboratory determinations, including RWC, lipid peroxidation, proline, and anthocyanins levels (Figure 2A), as well as from the physiological evaluation condition of plants by measuring gas exchange parameters (A, E, Gs, Ci) and chlorophyll fluorescence parameters (Fv/Fm, Y (PSII)) (Figure 2B).

The drought stress caused a decrease in leaf RWC in all the examined cultivars. There was an inversely proportional relationship between RWC level and MDA, anthocyanins as well as proline content in leaves of drought-stressed plants (Figure 2A). It means that drought stress induced the reduction of leaf hydration, which increased proline and anthocyanins content and lipid peroxidation level. However, the magnitude of these changes was different in the examined cultivars. There was a strong positive correlation between the MDA and the anthocyanins content, and also a positive, but weaker, correlation between the MDA and proline as well as the anthocyanin and proline content.

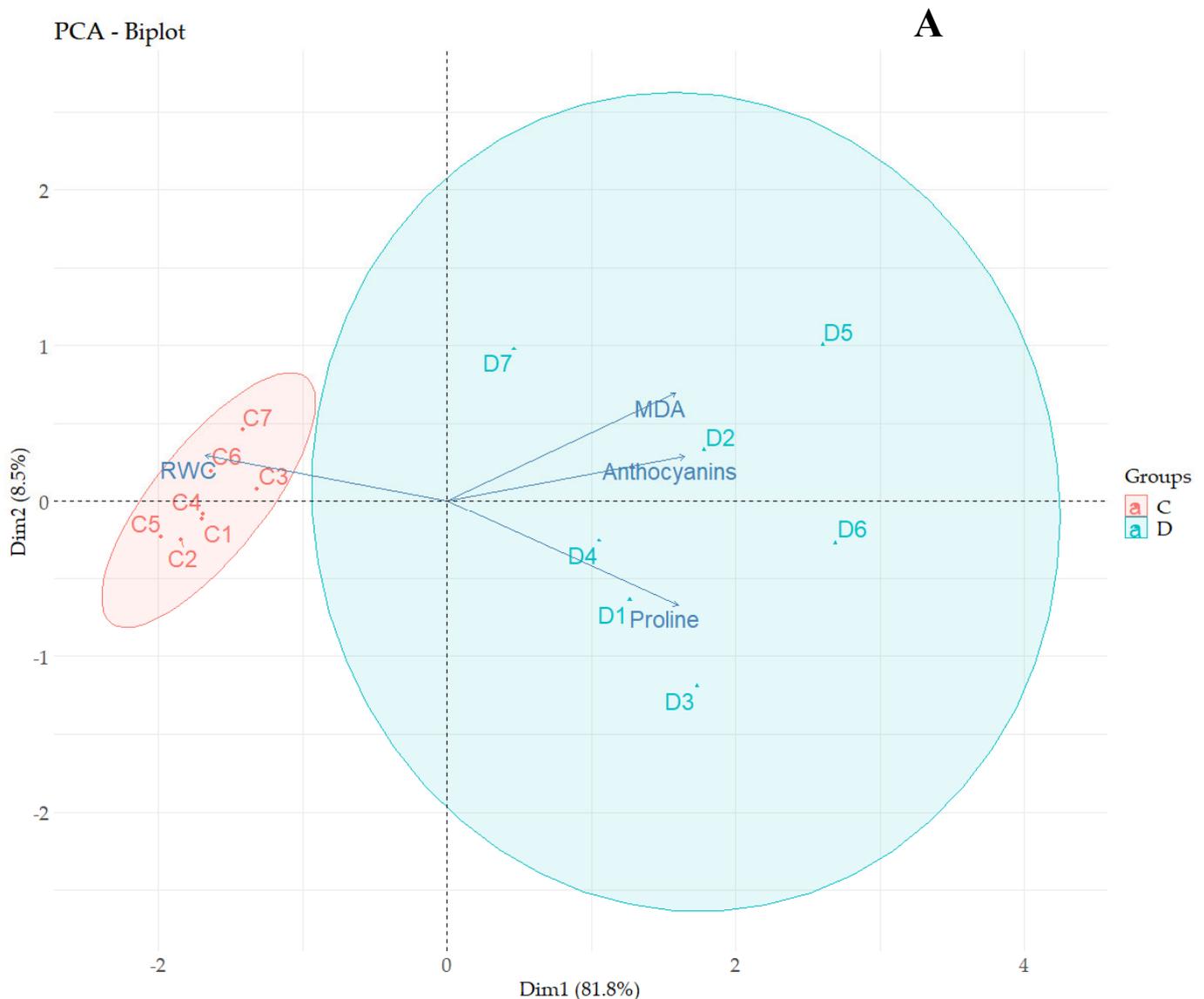


Figure 2. Cont.

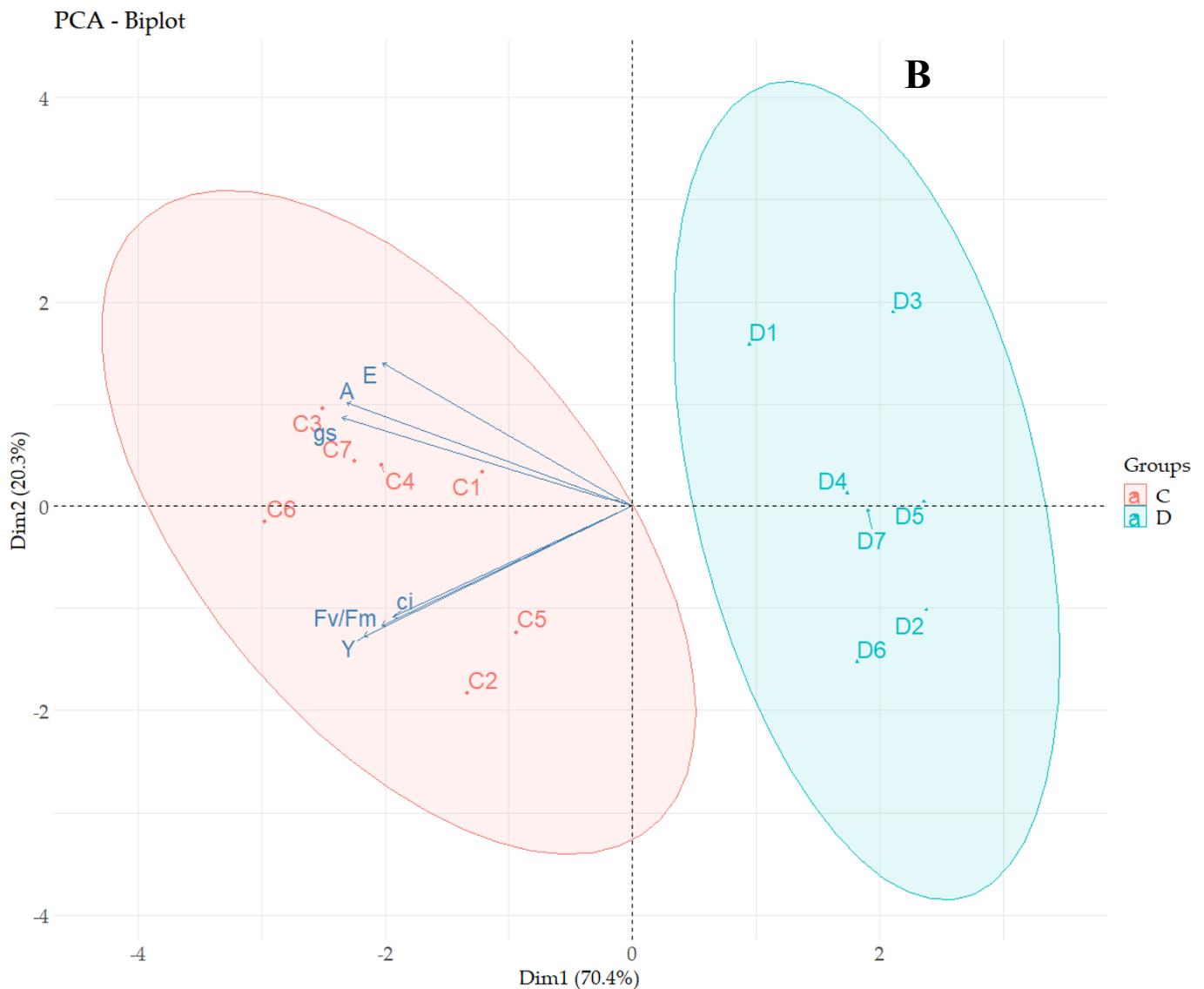


Figure 2. Projection of the variables on the component plane (1 × 2) of the effect of drought stress on: (A) relative water content (RWC), lipid peroxidation index (MDA), anthocyanins, and proline accumulation; (B) photosynthesis-related parameters (CO₂ assimilation level (A), transpiration rate (E), stomatal conductance (Gs), intercellular CO₂ concentration (Ci), maximum PSII quantum yield in the dark-acclimated state (Fv/Fm), PSII quantum yield in the light-acclimated state (Y (PSII)). C1–control ‘Franckenkorn’; C2–control ‘Oberkulmer’; C3–control ‘Badengold’; C4–control ‘Ostro’; C5–control ‘Divimar’; C6–control ‘Schwabenkorn’ and C7–control ‘Bogatka’. D1–7–stressed cultivars, in the same order.

A strong positive correlation was shown between the intercellular CO₂ concentration (Ci) and the Fv/Fm and Y (PSII) parameters (Figure 2B). The second group of variables showing a positive correlation but with a lower strength were: CO₂ assimilation (A), stomatal conductance (Gs), and transpiration (E). At the same time, both groups of variables showed a positive but definitely weaker correlation with each other. It should be noted that spelt wheat ‘Franckenkorn’ cultivar showed the lowest response to drought on gas exchange parameters. Both well-watered (C1) and drought-stressed (D1) ‘Franckenkorn’ plants were placed much closer to each other compared to the remaining tested cultivars. Similarly, only a slight effect of drought on these parameters was observed for the ‘Badengold’ spelt cultivar (C3 and D3).

3.8. Correlation Analyses

Figure 3 presents the correlation coefficients between RWC, lipid peroxidation level, anthocyanins, and proline content in leaves for each of the examined wheat cultivars.

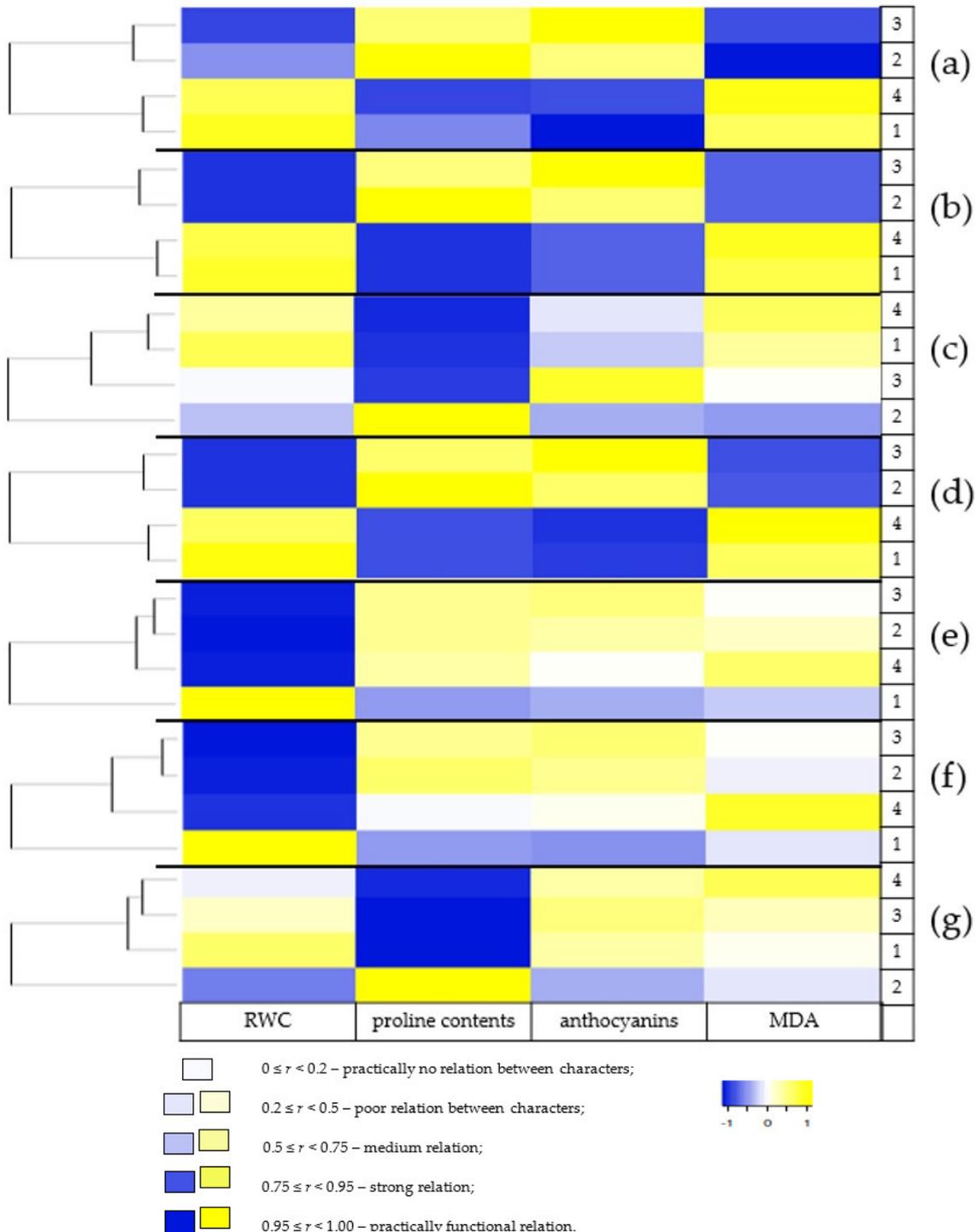


Figure 3. Correlation coefficients between features analyzed for stressed cultivars; (a) 'Franckenkorn'; (b) 'Oberkulmer'; (c) 'Badengold'; (d) 'Ostro'; (e) 'Divimar'; (f) 'Schwabenkorn' and (g) 'Bogatka'. Features compared: relative water content (RWC), proline contents and anthocyanins, lipid peroxidation index (MDA). Interpretation of Pearson's linear correlation coefficient: $0 \leq r < 0.2$ —practically no relation between characters; $0.2 \leq r < 0.5$ —poor relation between characters; $0.5 \leq r < 0.75$ —medium relation; $0.75 \leq r < 0.95$ —strong relation; $0.95 \leq r < 1.00$ —practically functional relation.

To elucidate the possible response mechanisms of spelt wheat to drought stress, correlations of RWC with other physiological parameters of tested cultivars under drought stress conditions were analyzed. The RWC average for cultivars showed a negative correlation with proline and anthocyanins contents ($r = -0.52^{**}$, -0.46^{**} , respectively) under drought stress conditions, suggesting that decreased relative water content in leaves may have induced proline accumulation. In addition, MDA, a marker of stress-induced damage, was significantly negatively correlated with proline content ($r = -0.21^{**}$), which may imply the enhancement of the defensive role of proline. Similar dependences were observed among the cultivars tested, and a significantly negative correlation between RWC and proline content was found in spelt wheat 'Oberkulmer' ($r = -0.95^{**}$), 'Divimar' ($r = -0.97^{**}$) and common wheat 'Bogatka' ($r = -0.99^{**}$). However, the above dependence was not observed in 'Franckenkorn', in which this correlation was weakly negative (-0.37^{*}). Interestingly, in this cultivar, RWC was strongly negatively correlated with anthocyanins ($r = -0.88^{**}$). This response suggests a relatively important role of anthocyanins in the anti-oxidative defense. Therefore, the relative water content, proline, and anthocyanins content might be associated with drought resistance in spelt wheat.

4. Discussion

Plants are known to respond to drought stress with a cascade of responses at molecular, biochemical, and physiological levels [37]. The essential physiological response is a decrease in photosynthetic efficiency, the result of stomata closure. The stomata closure process is initiated rapidly (within 1 to 2 min) after the occurrence of the causative agent and is completed within 5 min. Maintaining the balance between CO₂ exchange and transpiration is necessary to maximize the CO₂ assimilation in the photosynthesis and, at the same time, to reduce water loss [38]. The reasons for reduced photosynthesis efficiency are, among others, the reductions of stomatal conductance and the CO₂ assimilation rate regulated practically concurrently [39]. These dependencies were presented in this study as the experimental results revealed a positive correlation between parameters A, Gs, and E.

The obtained results showed the differences between the examined cultivars of spelt wheat and between spelt and common wheat in response to drought. Spelt wheat 'Franckenkorn' and 'Badengold' cultivars were characterized by the weakest impact of drought on gas exchange parameters. In turn, the strongest response to drought was characteristic of the 'Schwabenkorn', also characterized by the greatest decrease in RWC.

It was shown that in a transgenic wheat genotype that contains a selective gene that encodes proline biosynthesis, the decrease of the photosynthetic rate was lower than in the wild (not transformed). This was accompanied by an increase in the concentration of proline in drought conditions [40]. Similarly, in the presented research, 'Franckenkorn', characterized by a significant accumulation of free proline and anthocyanins, and a relatively modest decrease in the level of RWC, also showed the smallest decrease in the intensity of photosynthesis in drought conditions. 'Franckenkorn' was also characterized by a much lower level of lipid peroxidation (MDA), which may indicate the role of proline and anthocyanins in alleviating damage and maintaining photosynthetic activity. Chen and Li [41] confirmed that a high concentration of proline in the suspension cells of transgenic plants prevented lipid peroxidation of the cell membranes. They emphasized that proline plays an important role in the protective antioxidant system, and its presence reduced the oxidative damage in transgenic wheat lines, resulting in comparatively greater plant resistance to drought stress [41]. The opposite relation was found in the 'Schwabenkorn', in which a strong negative correlation between RWC and the accumulation of free proline was proved. This variety was characterized by the lowest RWC during drought with a relatively high accumulation of proline and a significant increase in the level of lipid peroxidation. In addition, the stress resulted in the greatest decrease in CO₂ assimilation in this variety.

The level of free proline in wheat leaves increases due to drought [42,43] and wheat cultivars with higher free proline content and lower lipid peroxidation (MDA) show better performance under drought at various stages of growth [44,45]. As early as 1987,

Raghavendra and Reddy [46] reported that proline acts as an osmoregulator and plays a protective role for the photosynthetic apparatus. In turn, lipid peroxidation expressed as MDA content indicates drought stress damage at the cellular level [47]. In this study, the increase in proline and MDA content was variable and indicated differences in the ability to cope with stress in tested cultivars.

It seems that the significant accumulation of anthocyanins correlated with the lack of oxidative damage was an element of the drought stress coping strategy in the 'Franckenkorn' cv. These results are consistent with the results obtained in the studies on wheat [21]. The authors of these studies showed that increased anthocyanins levels, as a component of drought coping strategy, prevented the increase in ROS levels in wheat leaves subjected to drought stress. The authors point out that anthocyanins facilitate plant survival when competition is high and resources are limited, potentially making them ideal physiological inducers of resilience and adaptation, mediated by their light screening properties, in a unified stress resistance mechanism.

According to Pietragalla and Mullan [48], plant genotypes with the ability to maintain full leaf turgor under drought minimize the effects of the stress, allowing for the proper course of turgor-dependent processes, such as plant growth and the activity of the stomata and complexes of photosystems I and II. This study reported differences in the RWC among the tested cultivars. The drought stress caused a moderate decrease in RWC, the highest in the 'Schwabenkorn' cultivar and the lowest in the 'Bogatka' cultivar. Similar results were obtained by Keyvan [49], who also showed significant differences between wheat cultivars in the level of leaf hydration (RWC) and free proline accumulation under drought stress. Based on the increased content of free proline and the inhibition of chlorophyll fluorescence, Dib et al. [50] divided the analyzed durum wheat cultivars into drought-resistant and -sensitive.

The reduced assimilation of CO₂ caused by drought may lead to a disturbance between the supply and demand in the assimilation power of ATP and NADPH in the course of photosynthesis. Under such circumstances, or if the antenna complexes supply too much energy under drought conditions, the generation of reactive oxygen species may occur. Consequently, this leads to an irreversible degradation of the components of the photosynthetic apparatus and lipid peroxidation. The resulting metabolic situation forces the plant to dissipate the excess energy absorbed by chlorophyll in several ways, one of which is increased fluorescence [51,52].

The measurements of chlorophyll fluorescence parameters are subject to various modifications depending on the type, duration, and intensity of the stress; therefore, it allows the detection of changes in PSII which result from plant-perceived stress, even before any visible symptoms of damage manifest. Chlorophyll fluorescence parameters can be used as a criterion for selecting cultivars and species more resistant to stress factors [53]. As early as 1985, Havaux and Lannoye [54] used the fluorometric method to select drought-sensitive and resistant durum wheat cultivars. Our results show that spelt wheat cultivar ('Franckenkorn') characterized by the lowest decrease in stomatal conductance and gas exchange parameters exhibited a relatively large decrease in the fluorescence parameters. 'Franckenkorn' maintains open stomata, relatively high CO₂ assimilation, and transpiration with a simultaneous loss of leaf chlorophyll content and decline of PSII quantum yield in the light-acclimated state. It could be a defense mechanism consisting of the dissipation of energy absorbed by chlorophyll or a result of lost carboxylation capacity and/or increased thermal dissipation.

A decrease in the maximum fluorescence (F_m) and the maximum PSII quantum yield in the dark-acclimated state (F_v/F_m) was also observed in this cultivar. Gilmore and Björkman [55] showed that F_m attenuation accompanies an increase in energy dissipation. Zlatev [56] also revealed a decrease in the maximum fluorescence (F_m), the maximum PSII quantum yield in the dark-acclimated state (F_v/F_m), as well as parameters taken under light: Y (PSII) and ETR in two winter wheat cultivars under drought stress.

Only severe water deficit (RWC decrease of more than 30 percentage points) in the leaf is the limit below which biochemical processes are significantly impaired [57]. There-

fore, the observed decreases in the values of chlorophyll fluorescence parameters in the 'Franckenkorn' imply a slowdown in electron transport rate and the maximum PSII quantum yield in the dark-acclimated state. This could indicate the triggering of a drought defense mechanism, due to which high photosynthetic efficiency is retained. Water stress can potentially lead to an increased susceptibility to photoinhibition if excess excitation energy cannot be safely dissipated [58]. Genty et al. [59] demonstrated that the concentration of open PS II reaction centers and the efficiency of excitation capture by PSII centers determine the quantum yield of non-cyclic electron transport, and that the deactivation of excitation within PS II complexes by non-photochemical processes must influence the quantum yield of non-cyclic electron transport. Regulation of thermal and photochemical de-excitation pathways, together with the PSII recovery system, all contribute to a photoprotective system that prevents photodamage to the photosynthetic apparatus [60]. It has also been suggested that the inactivation of PSII, which causes reversible damage, can prevent the largely irreversible damage to PSI [61]. Lack of a stomatal reaction likely caused a lesser reduction of CO₂ assimilation and transpiration in 'Franckenkorn' compared to the other analyzed cultivars. However, it led to partial degradation of chlorophyll and a reduction in the electron transport rate and the maximum photochemical yield of PSII. Drought stress inhibits the synthesis of chlorophyll, leading to a decrease in the content of chlorophyll-binding proteins a and b, leading to a preferential reduction of the PS II-associated light-gathering pigment protein [62]. Therefore, the 'Franckenkorn' cultivar is a very important material for studying the mechanism of drought resistance. As shown in this study, genetic variations of drought stress resistance in spelt wheat cultivars might be largely related to the induced changes in some important physiological parameters, especially relative water content, proline level, and the antioxidant defense system.

5. Conclusions

The obtained results indicate differences in the response to drought in the examined varieties of spelt. Drought caused a water deficit in the leaves, which influenced metabolic changes and physiological activity. The 'Schwabenkorn' variety turned out to be the most susceptible to drought. This sensitivity was manifested by the greatest decrease in the level of RWC, stomatal conductivity, and CO₂ assimilation, as well as an increase in the level of MDA (damage). The high accumulation of proline in this cultivar could be caused by a significant decrease in the level of RWC (up to 54%), indicating a strong water deficit in the leaves. The 'Franckenkorn' variety was found the most resistant, showing a significantly lower reduction of RWC than 'Schwabenkorn' (up to 72%), no damage to cell membranes (no increase in the MDA level), and the smallest reduction in stomatal conductivity and CO₂ assimilation. The accumulation of anthocyanins and proline appears to have played a role in this case. The level of free proline in this cultivar under drought conditions was similar to that in the sensitive 'Schwabenkorn', and the level of anthocyanins was significantly higher. These changes may have contributed to reduced RWC (osmotic adjustment) and limited damage (lipid peroxidation-MDA).

The obtained results also indicate that, among the spelt cultivars, 'Badengold' may be considered more resistant to drought than the others, probably due to high proline accumulation responsible for alleviating dehydration and protecting against lipid peroxidation (slight MDA increase). Consequently, the extent of inhibition of the CO₂ assimilation rate was low. These results suggest that changes in water content in leaves, antioxidant defense system, and the action of photosynthetic apparatus may contribute to the variations in drought tolerance between cultivars of spelt wheat. The 'Franckenkorn' and 'Badengold' cultivars, identified as drought-resistant based on physiological parameters, and the 'Schwabenkorn' cultivar, identified as susceptible, may be considered for plant breeding programs aimed at enhancing drought resistance in economically important crops such as common wheat.

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