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Comparison of Short-Term Physiological and Biochemical Effects of Drought Stress on Two Wheat Cultivars

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HIGHLIGHTS

- Proline is 2.5 times higher in Bejostaja-1 with respect to Tosunbey at the highest PEG-6000 concentration
- The GSH pool is 10 times, total GST activity is 3 times higher in Bejostaja-1 at severe drought
- Bezostaja-1's protein profile changed dramatically, especially at peak drought pressure
- GSH, GST activity and protein profile are useful for evaluating drought resistance.

Abstract: Drought stress, which is becoming more frequent and severe, restricts grain production. It is critical to choose wheat (*Triticum aestivum* L.) cultivars that are drought-resistant enough in progressively arid climates. Early physiological and biochemical responses were measured by applying drought stress to susceptible Bezostaja-1 and tolerant Tosunbey wheat cultivars with three different PEG-6000 concentrations to understand the reliability, selectivity and practicality of tested parameters. Depending on the elevated drought, lengths, fresh-dry-turgor weights, relative water content of both root and stem decreased, and chloropyll amounts increased similarly but in different dimensions in both cultivars. While a decrease was observed in the total thiol content of both, it was determined that this value was 10 times higher in Bezostaja-1 cultivar. While proline amounts peaked at the most severe drought, this value was found to be 2.5 times higher in Bezostaja-1 than in Tosunbey. The protein content is 35% higher in Bezostaja-1. In contrast to glutathione S-transferase theta1 (GSTT1) isozyme, total GST activity increased 53.1% in Bezostaja-1 and 33.6% in Tosunbey, compared to control. This average values are about three times higher in Bezostaja-1 compared to Tosunbey. Of the experimental groups whose band profiles were generated by SDS-PAGE and compared with UPGMA and neighbor-joining methods, moderate to high drought samples of Bezostaja-1 showed explicitly different results and possibly a metabolically distinct response from all others. Besides

frequently used ones, total thiol content, total GST activity and changes in the protein bands could be used as useful parameters in the selection of drought-resistant cultivars.

Keywords: drought stress; *Triticum aestivum* L.; resistant cultivar; antioxidative protection; PEG-6000.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.), which is the most cultivated rain-fed crop in the world, is a very important plant that is grown in approximately 30% of the entire agricultural area and provides almost one-fifth of the average daily caloric needs of modern humans [1]. There are ten major wheat-producing regions; Europe, Russia, Canada, USA, Ukraine, Australia, Kazakhstan, Turkey, Argentina and Brasil; and, they constitute 54% of the global wheat-growing area, resulting 57% of all wheat production, and account for more than 92% of wheat exportation [2]. Except for the last two, all these regions are under threat of drought; and, the situation is reported to be much worse in the near future [3]. For this reason, while seeking a solution to climate change and the incidence of drought, it is even more important to plant wheat cultivars that can be grown in arid conditions and provide high yields, and to reveal the advantages of these wheat cultivars through scientific studies.

Understanding the basics of physiological and biochemical mechanisms that enable wheat, especially its hardy cultivars, to cope with different environmental factors such as drought is of vital importance [4]. The effects of global climate change on wheat agriculture and yield have been studied with a wide variety of methodologies for a long time; however, there are few publications on adaptation strategies. Among these adaptation strategies, the different cultivars of the countries and their cultural practices have a separate place and importance. Moreover, the level and content of drought resistance shown by wheat cultivars, also, vary according to the developmental stage; therefore, as an additional screening criterion to determine the adaptability and resistance levels of different wheat varieties, as well as their usability and benefit in agricultural areas against drought, observing the antioxidative protection of the same cultivars at the early seedling stage under drought would undoubtedly add new dimensions to the studies on this subject and the evaluation of the results.

Most of the total injury in wheat, as in all other plant species under drought pressure, is due to oxidative damage at the cellular level. However, more drought-resistant cultivars are known to have metabolic advantages that provide a higher level of antioxidant protection. Thiols and glutathione (GSH) are considered two crucial groups of low molecular weight metobilites having roles in the prevention of oxidative stress by functioning as detoxicants and antioxidants in plants, as well as in wheat species [5]. In related metabolism, glutathione S-transferases (GSTs, E.C.2.5.1.18) perform the conjugation of GSH to various electrophilic substrates, besides contributing to overall cellular defense against drought stress at many other points [6].

Drought stress causes changes in many biochemical pathways, including growth and stress defense mechanisms, and thus significant changes in the protein profile of wheat [7]. There are two important groups of plant responses to water shortage: the first group is to produce and accumulate osmolytes such as proline, which will allow it to make osmotic adjustments, inhibit membrane disintegration, and inactivate certain enzymes [8]. Up-regulation of some drought-related genes and protective proteins such as elements involved in signal transduction, transcription factors, antioxidants and various ROS suppressors could be considered as the second group response [9]. The comparison of these two groups of responses at different developmental stages, as well as the monitoring of protein content and the comparison of cultivars with varying degrees of drought tolerance, provides economic and ecological benefits.

In this study, the physiological and biochemical responses of tolerant Tosunbey and susceptible Bezostaja-1 wheat cultivars to three different stages (low-moderate-high) of drought stress were evaluated, first, to understand the reliability, selectivity and practicality of those parameters, especially GST enzyme activities and protein band comparisons; and, second, to cross-compare those varieties for their initial success against water deficit in the short-term. For this purpose, two wheat cultivars were treated with three different degrees of water deficit by 5%, 10%, and 20% PEG-6000 applications. Their physiological and biochemical responses as changes in length, weight (dry, fresh, and turgor weights), relative water content, photosynthetic pigments (chloropyll-a, chlorophyll-b, and total carotenoids), proline content, relative protein content, total thiols and GST activities were compared by using values derived from respective control groups. Additionally, the protein band patterns of experimental groups were, also, compared by SDS-PAGE analysis and used to construct UPGMA and neighbor-joining based phylograms.

MATERIAL AND METHODS

Wheat seeds, cultivation and stress application

Tosunbey bread wheat (*Triticum aestivum* L.) seeds, which is a Turkish cultivar, were obtained from the Field Crops Central Research Institute of the Ministry of Agriculture and Forestry of the Turkish Republic. Bezostaja-1 bread wheat (*Triticum aestivum* L.) seeds were obtained from the Transitional Zone Agricultural Research Institute of the Ministry of Agriculture and Forestry of the Turkish Republic. The Tosunbey cultivar is relatively resistant to drought stress with respect to the Bezostaja-1 cultivar, which was known as drought-susceptible at early growth stages [10]. The suitable megaenvironment of Tosunbey cultivar was stated as ME12 which is characterized by low rainfall (annual precipitation < 500mm) and drought constraint [11]. On the other hand, the suitable megaenvironments for Bezostaja-1 cultivar are ME-10 and ME-11, for which high rainfall and/or irrigatation are assigned with no drought threat [12].

Tosunbey and Bezostaja-1 wheat seeds were weighed as 3 grams for each experimental repeat; and, incubated in 3% NaOCl in an orbital shaker incubator, for 5 minutes at 24°C. Seeds were washed with distilled water; and, planted in cups containing Hoagland solution at pH 5.7 - 5.8, as 10 seeds in each. After sowing, wheat seeds were allowed to grow in a controlled climate room for 7 days in a 12-hour light and 12-hour dark cycle. The decreasing Hoagland solutions in the cups were refreshed at regular intervals to ensure the continuity of growth.

On the 7th day of growth, Hoagland solutions were discarded and the germinated wheat samples were treated with different concentrations of PEG6000 solutions of 5%, 10%, and 20%, which were prepared in Hoagland for 5 days to induce drought stress. Fresh Hoagland solution was added to the control groups. In this way, the whole set of one experimental group contains 3 groups of PEG6000 applications, for each of which there were 5 cups, and a control group that included 5 cups, too. It was the same for each cultivar, and the whole experiment was repeated independently at four different dates. A total of 32 sample groups (each had 5 cups) were included in the study.

Measurements of physiological parameters

Tosunbey and Bezostaja-1 wheat cultivars exposed to different polyethylene glycol concentrations were measured in terms of dry weights (DW), fresh weights (FW) and turgor weights (TW) as well as stem and root lengths. In addition, the relative water content of the stems and roots was also calculated. All these values were compared with those obtained for the control group. Fresh weights were measured on a precision scale; turgor weights were measured by keeping the stems and roots in distilled water for 24 hours, then toweled and weighed on a precision balance; and dry weight measurements were completed by keeping the samples in a 72°C incubator for 48 hours and weighing them on a precision balance. The relative water content (RWC) of the plant was calculated using the values of wet and dry weights and turgor weights according to Equation 1 [13].

$$RWC\ (\%) = [FW\text{-}DW] / [TW\text{-}DW] \times 100 \quad (1)$$

Spectrophotometric determination of photosynthetic pigments

Repeated measurements were carried out using different solvents for the determination of photosynthetic pigments. For chlorophyll-a and chlorophyll-b, 95% ethanol, 100% methanol, 100% acetone, and 80% acetone were used. For the determination of the total carotenoid amount, 80% acetone was used.

50 mg of ground samples were weighed and 2 ml of each solvent were added. After incubation at 4°C overnight by occasional vortexing, the tubes were centrifuged at 15000g for 5 min. The absorbance values of the supernatant were measured at predetermined wavelengths by a double-beam spectrophotometer (PG Instruments T80+ UV/VIS) to be used in previously reported equations [14] for calculation.

Determination of proline content

The determination of proline is based on the colorimetic feature of the reaction of this amino acid with the chemical ninhydrin [15]. A total of 400 mg of powdered wheat sample was weighed and 40 mg of PVPP was added. After adding 1.5 ml of 3% sulfosalicylic acid and vortexing, it was centrifuged at 15000 g at +4°C for 6 minutes. The supernatant was collected and mixed with acid ninhydrin, acetic acid (96%), and sulfosalicylic acid (3%) at a volume ratio of 1:2:2:1 and incubated at 96°C for 1 hour. 1 ml of toluene was added to the tubes and centrifuged for 5 minutes at 15000g at 4°C. Absorbance values were read in quartz cuvettes using a spectrophotometer set at a wavelength of 520 nm. The results were evaluated and the final

concentrations were calculated by the slope of the standard curve, which was constracted by repeating the procedure for a series of ten dilutions of pure proline in the 1 mM–1 M interval.

All the spectrophotometric measurements of photosynthetic pigments and proline were performed as dublicated tubes and their blanks, by a spectrophotometer (PG Instruments T80+ UV/VIS). All tests were repeated at three different dates for strong statistical inference.

Homogenization of wheat samples

The leaf samples were grinded by mortar and pestle with liquid nitrogen and a fine powder form was collected and stored at -80°C freezers until the day of experiment. 0.2 g of powdered sample were mixed with 0.02 g of PVPP; and, 2 ml of cold homogenization buffer (0.1 M Tris HCl buffer at pH 7.4, containing 0.07% (v/v) 2-Mercaptoethanol, 5% (w/v), 2 mM of EDTA, 0.5% Nonidet P40, protease inhibitor cocktail which is a mixture of AEBSF, E-64, Bestain, Pepstatin, Leupeptin, and 1,10-Phenanthroline) were added to each tube. Homogenization was carried out on ice using Ultra-Turrax T25 at 13500 rpm for 4 times at 15 second intervals (15 seconds x 4). The homogenate was centrifuged at 12000g for 15 minutes at 4°C, and the supernatant was collected, aliquoted, and stored at -80°C freezers for future testing.

Total thiol content and GST enzyme activities

Protein concentrations of the homogenates were detected by the Bradford method. In an ELISA Plate Reader system (Thermo, MultiskanTM FC Microplate Photometer) adapted format, commercially available Bradford reagent was used as defined by the supporter.curve was formed by running the same procedure for a series of dilutions of BSA (0.1-1.0 mg/ml).

The total thiol content of each sample was determined by the method of Sedlak and Lindsay [16], which was later adapted for the ELISA Microplate Reader system [17]. The reaction that takes place during the determination of the thiol content is based on the binding of the free thiol group to the DTNB molecule and the reduction of the molecule by breaking the double bond. All the samples and conrol groups were evaluated and the relative total thiol content, which was also used to be a measure for the GSH pool that constitutes a large portion of all thiols, was calculated by using the slope of a standard curve prepared for a series of GSH solutions with increasing concentrations from 1 mM to 10 mM.

Total GST activity measurements were completed using CDNB as a general substrate, at 340nm, using the method of Habig and coauthors [18] optimized for the ELISA Microplate Reader system [17]. The enzyme was added to each well containing 100 mM phosphate buffer adjusted to pH 7.4, 1 mM GSH, and 1 mM CDNB to begin the reaction. For the reaction wells, protein concentrations were in the range of 48-150 g/ml for Tosunbey and 9.6-30 g/ml for Bezostaja. Total GST specific activity was calculated according to Equation 2.

$$\text{Specific Activity} = ((dA/dt) / 9.6\ mM^{-1}cm^{-1}) \times DF \times (1/mg\ protein\ ml^{-1}) \quad (2)$$

In the equation, dA/dt represents the change in absorbance per minute, and, DF represents the dilution factor.

The specific activities of GST Theta isozyme was determined by the method of Habig and coauthors [18] optimized for the ELISA Microplate Reader system [19]. Plant homogenates were added to each microplate well containing 100 mM phosphate buffer adjusted to pH 6.5, 0.5 mM GSH, 0.25 mM EPNP, and the enzymatic reaction was started. Protein equivalent to that of total GST activity was added to the wells. Specific activity values were calculated using Equation 2, but 0.5 $mM^{-1}cm^{-1}$ was used as the extinction coefficient.

All the enzymatic measurements were completed with triplicated wells and one respective blank well. All tests were repeated at three different dates, independently, to provide essential statistical inference.

SDS-PAGE and clustering issues

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) application was used to observe the band profiles of the samples. 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad) were used. PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) marker was added in a volume of 5 µl. The samples were loaded into the wells with 25 µl volume and 25 µg protein content. After running the gels by applying 100 V, they were stained with the Colloidal Coomassie Blue (CBB) method. Continuous shaking was applied in a mixture of methanol-acetic acid-distilled water (5:1:4 v:v) for half an hour at room temperature. It was then incubated for half an hour in methanol-acetic acid-distilled water (5:1:4) containing 0.1% Coomassie Blue R250. Afterwards, images of the gels were transferred to the computer after "destaining" by a mixture of methanol-acetic acid-distilled water (5:1:4 v:v) overnight. SDS-PAGE application

was repeated 4 times. Vilber Lourmat (V01-5346) with a basic camera attachment was used to get the gel images. The cluster analysis of groups was performed for bands on the gel image obtained after SDS-PAGE application by using the open source PyElph 1.14 software [20].

Statistical analyzes

Statistical analyses were performed using SigmaPlot.13 licensed software. In the comparison of experimental groups, the One Way ANOVA test and its extensions (Holm-Šídák, Dunn's, and Tukey's) were used to compare more than one group, and Mann-Whitney U was used in cases suggested by the software. Paired t-test was applied separately to compare some groups. A p value of smaller than 0.05 was considered statistically significant.

In the plots, the terms "α, β, and γ" were used to express the statistical value of the comparisons between the groups. "α" states a statistically significant difference with the control ($p<0.05$), "β" states a statistically significant difference with 5% PEG application ($p<0.05$) and "γ" states a statistically significant difference with 10% PEG application ($p<0.05$).

RESULTS

The change in physiological parameters due to increasing drought stress for each cultivar

Tosunbey and Bezostaja-1 cultivars were evaluated for their early physiological responses to mild to severe drought stress applied by the use of three different concentrations of PEG-6000 solution prepared in Hoagland solution, which imitates nutrient-rich soil solution. The dry weight (DW), fresh weight (FW), and turgor weight (TW) values were presented in Figure 1A and B for Bezostaja-1 and Tosunbey cultivars, respectively.

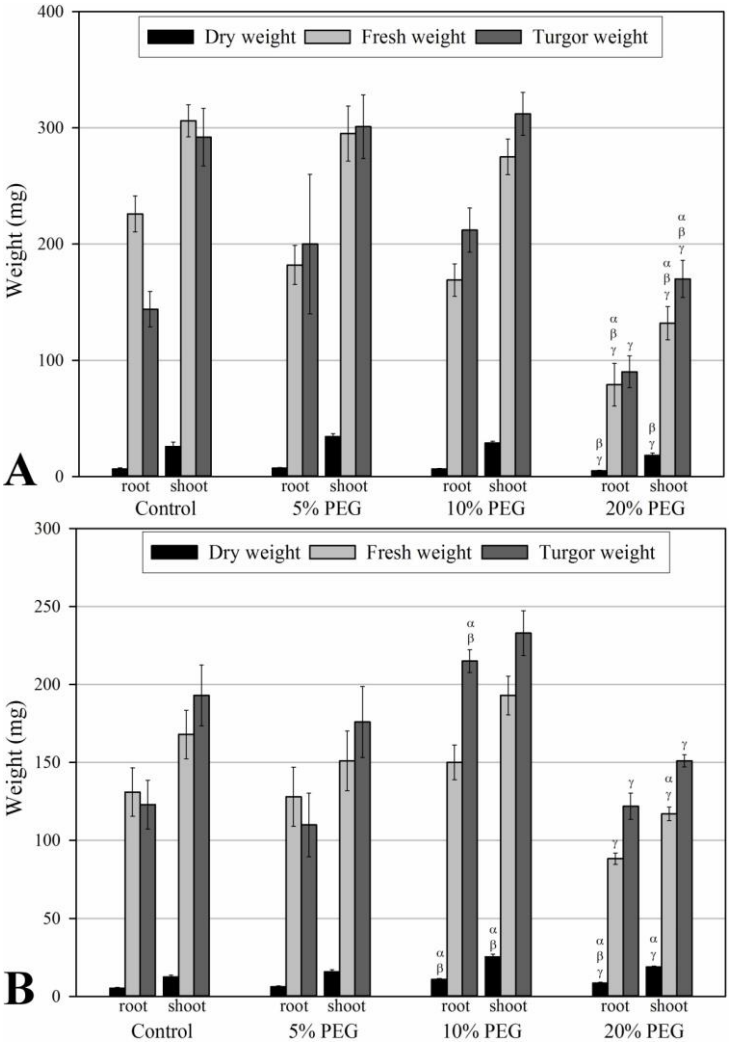

Figure 1. DW, FW and TW values of (A) Bezostaja-1 and (B) Tosunbey samples as presented for control, 5% PEG, 10% PEG and 20% PEG applications. Error bars demonstrate ±SEM (standard error of mean).

There were statistically significant decreases in FW and TW values, especially, in the 20% PEG group, at which the highest drought stress was achieved on wheat seedlings. For the intolerant Bezostaja 1 cultivar, with respect to the control group, the rates of decreases in DW, FW, and TW at the highest PEG concentration were 22.9%, 65.0%, and 37.4% for root; and, 29.1%, 56.9%, and 41.8% for shoot, respectively. In root and shoot samples of the resistant Tosunbey cultivar, for all three parameters, the slightly upward trend observed up to 10% PEG application ended with a dramatic decrease in 20% PEG application; in this respect, it showed a different change from the Bezostaja variety. If DW, FW and TW values of the 20% PEG application group are compared with the 10% PEG applied group's results, there are decreases of 22.0%, 41.1%, and 43.3% for root; and 25.6%, 39.4%, and 35.2% for shoot, respectively.

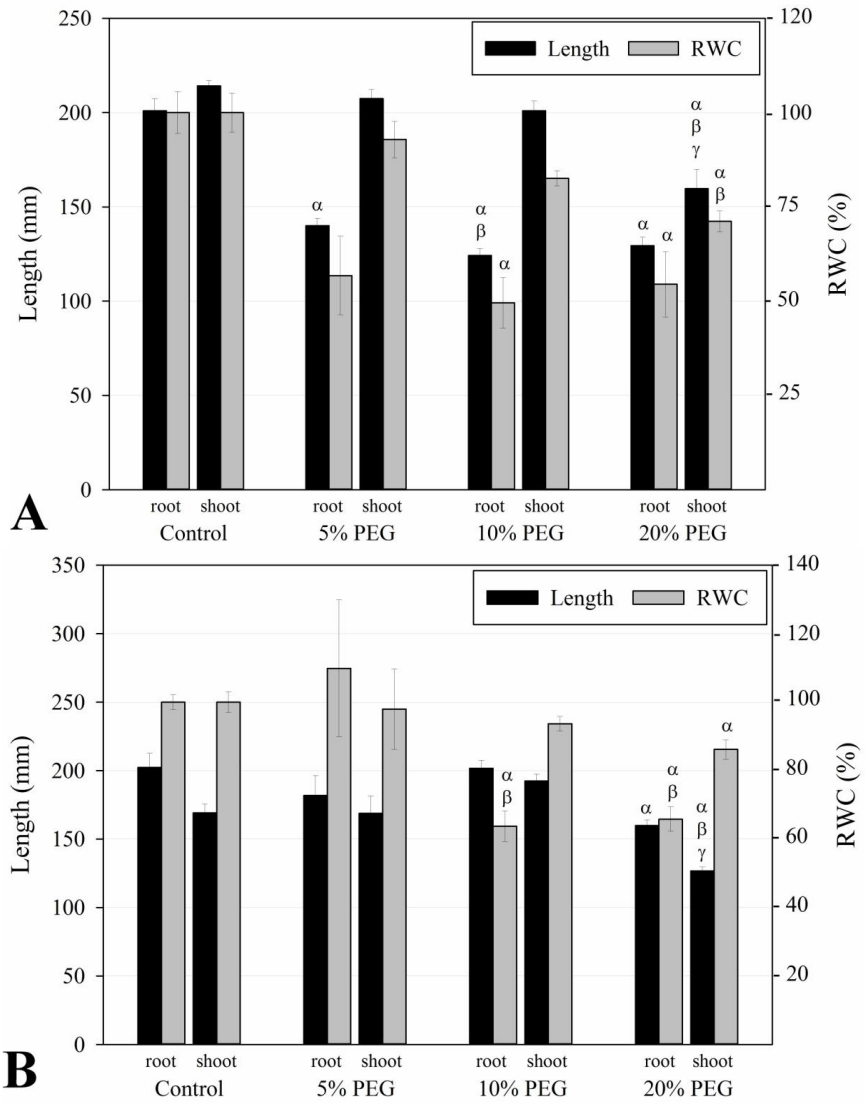

Figure 2. The calculated RWC of shoot and measured length values of both root and shoot samples of (A) Bezostaja-1 cultivar and (B) Tosunbey cultivar. Error bars demonstrate ±SEM (standard error of mean).

As an additional parameter for the evaluation of the effects of water deficit on wheat seedlings, root and shoot lengths of both cultivars were, also, recorded (Figure 2 A and B). Furthermore, DW, FW, and TW values were used to calculate the RWC, which is defined as a very relevant physiological measure of plant water deficit. Even if two different cultivars had the same leaf water potential, they could have different leaf RWC values, demonstrating a corresponding difference in leaf hydration, leaf water deficit, and physiological water status.

For Bezostaja-1 and Tosunbey cultivars, drought stress caused shorter seedlings, particularly at 20% PEG practice. Considering Bezostaja-1 seedlings, there were decreases in the values of length and RWC of 35.6% and 45.5% for root; and, 25.4% and 28.8% for shoot, respectively. The same situation is valid for the Tosunbey cultivar but with a smaller magnitude; i.e., there were reductions in length and RWC values of 21.0% and 34.22% for root; and, 25.1 and 13.8% for shoot, respectively.

The alterations in photosynthetic pigments stating the responses of cultivars in water deficit

Abiotic stress, such as drought, generally destroys leaf chlorophyll and some other photosynthetic pigments like carotenoids with an elongated exposure period. Although it is not a direct method of assessing the level of water deficit with great convenience, it is still used in many studies to compare the relative stress injury in terms of chlorophyll breakdown. However, the short-term response of those pigments to stress should be interpreted accordingly.

The chlorophyll-a (chl-a), chlorophyll-b (chl-b) and total carotenoid (car) contents of all experimental groups of both wheat cultivars were detected by a spectrophotometrical method by using three different solvent systems in extraction of pigments, to provide high reliability (Figure 3 A and B).

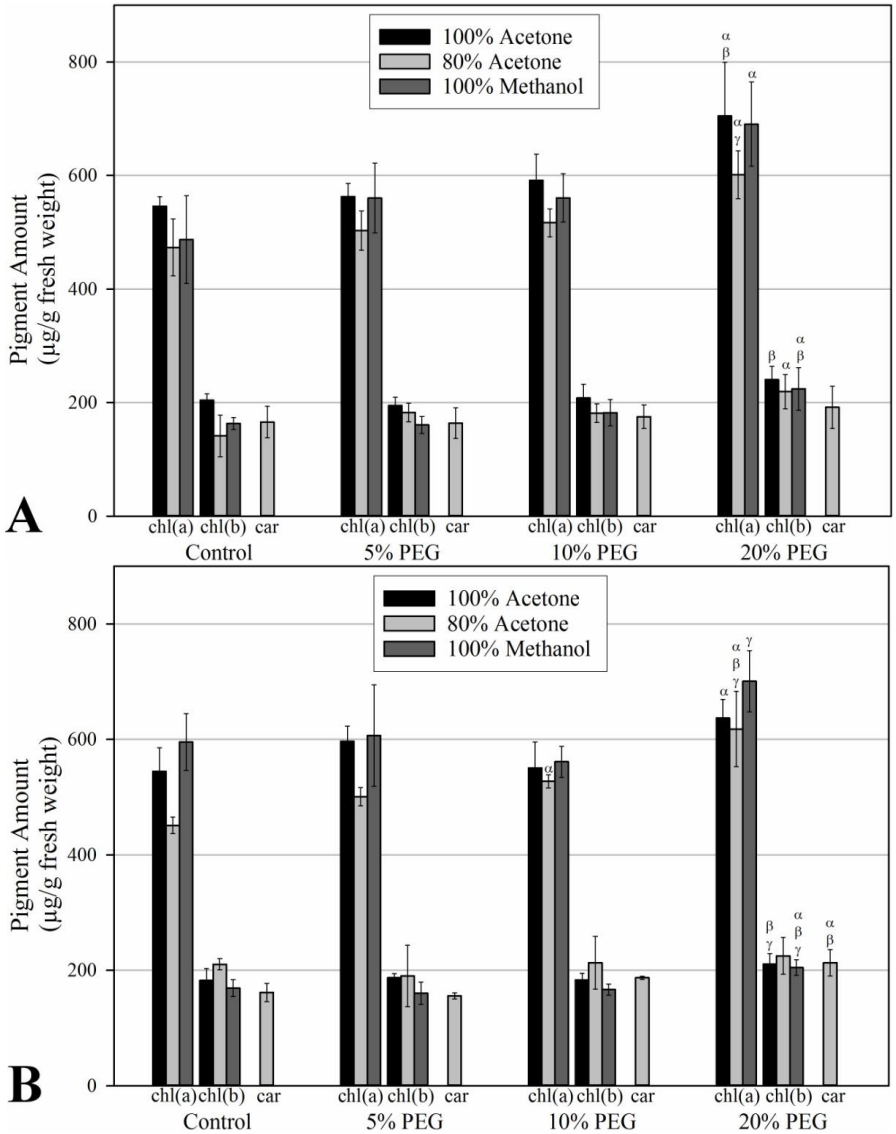

Figure 3. The changes in chl (a), chl (b) and car content of leaf samples of drought stressed (A) Bezostaja-1 and (B) Tosunbey seedlings, which were extracted by three solvents, independently: pure acetone, acetone:water mixture (4:1) and, pure methanol. Error bars demonstrate ±SEM (standard error of mean).

Concentrations of chlorophyll pigments were significantly elevated at the highest drought stress level at the end of a 5-day period. Before comparing the percentage increments, it should be noted that the calculated rates of increase in various solvents are quite different. It can be argued that this is due to the different properties and polarities of solvents used to dissolve chlorophyll pigments. However, it does not change the fact that the short-term physiological response of both cultivars was to increase photosynthetic pigments due to increased drought stress.

The rate of change for chl (a) of Bezostaja-1 when control and 20% PEG-administered groups are compared, for all three solvents, was 32.6% increase on average. The same comparison for chl (b) yielded an average percentage increment of 36.7%. There were increases in concentrations of chlorophyll pigments

with severe drought stress in Tosunbey experimental groups like Bezostaja-1, but the magnitude of changes was significantly smaller. While for chl (a) content, the increase in the 20% PEG group was detected as 24.1%, on average of all solvent trials, chl (b) concentration was elevated 14.4% on average. There is no statistically significant change in the total carotenoid content of experimental groups belonging to the Bezostaja-1 cultivar. Unlike the drought-susceptible one, the car content of 20% PEG applied susceptible Tosunbey seedlings was calculated as 31.9% increased with respect to control.

The variations in biochemical markers with respect to drought conditions

Drought stress often causes an imbalance between reactive oxygen species (ROS) and the antioxidant system. Plants, like many other organisms, have an array of defense mechanisms, including glutathione and glutathine S-transferases (GSTs) against those ROS. Because GSTs account for approximately 2% of all soluble proteins in wheat seedlings [21], increased GST isozyme activity has been linked to the presence, intensity, and duration of abiotic stress [22].

The changes in specific activities of total cytosolic GSTs and GST theta 1 (GSTT1) isozyme were subjected to evaluate their potential contribution to the response of those drought tolerant and susceptible cultivars (Figure 4). Moreover, the total thiol contents of the samples were also determined to be used as a tool in assessing the changes in the GSH-GSSG pool of the plant samples. Last, in-between experimental groups, the variance in proline concentrations, which is one of the most dramatic water stress characteristics, was tracked by a spectrophotometric method (Figure 5).

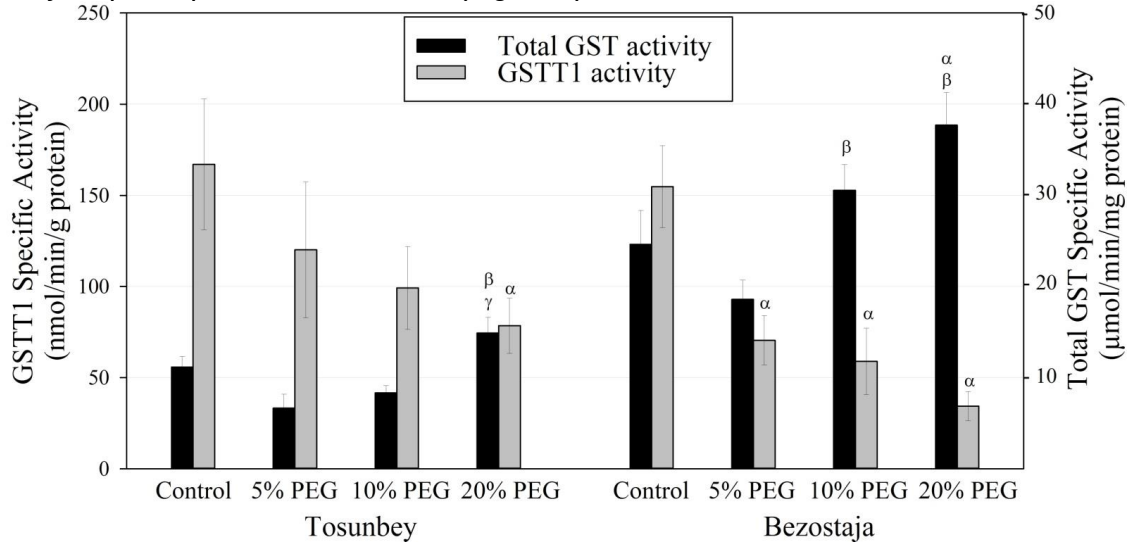

Figure 4. Total GST and GSTT1 isozyme specific activities of all experimental groups. Error bars demonstrate ±SEM (standard error of mean).

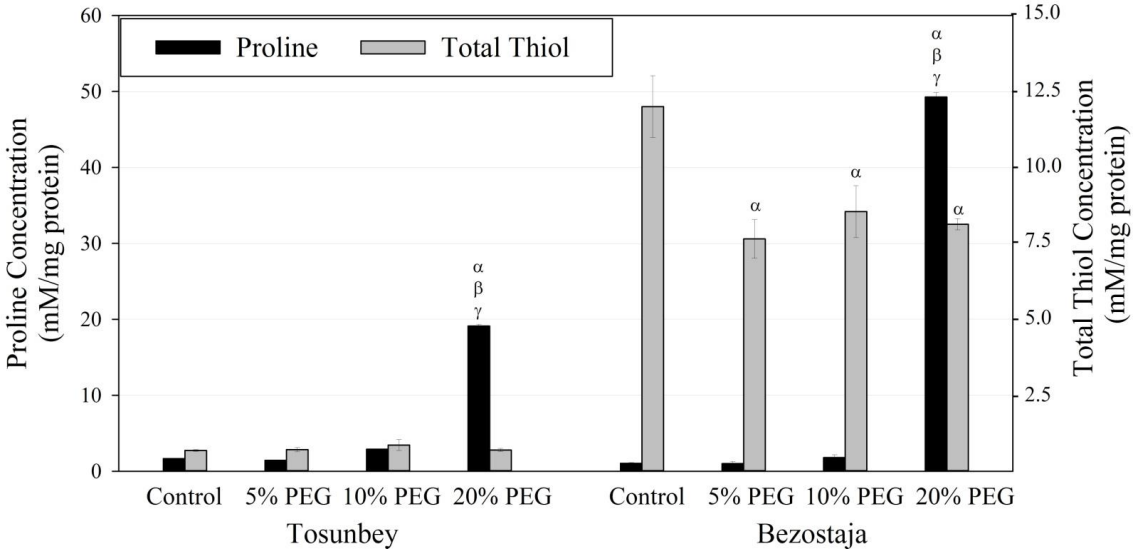

Figure 5. Proline and total thiol concentrations of all experimental groups. Error bars demonstrate ±SEM (standard error of mean).

Total GST specific activities of drought stress applied seedlings followed a similar pattern of change in two cultivars, so that, at 5% PEG group, there were decreases of 40.4% for the Tosunbey and 24.6% for the Bezostaja-1, although they were not statistically significant. The GST activities at the highest drought application reached higher levels ($p<0.05$) when they were compared with the values of the concerned controls; and, there was a 33.6% increase for the Tosunbey, and a 53.1% increase for the Bezostaja-1. In addition, the average total GST activities of the Bezostaja-1 cultivar were approximately 2.2-3.7 times higher than those of the Tosunbey cultivar at matching PEG groups. Notwithstanding elevated total cytosolic GST activities, the specific activities of GSTT1 isozyme, which were first detected in those kinds of studies in the literature, declined with elevated drought stress. Those values were 53.0% and 77.8% decreased at 20% PEG applications of the Tosunbey and the Bezostaja-1 cultivars, respectively.

There were no statistically significant changes in total thiol amounts detected in drought applied Tosunbey cultivars, with respect to the control. On the other hand, for the Bezostaja-1 cultivar, the percentage of decreases in total thiol concentrations at application groups was about 32.5%, on average. Moreover, the levels of total thiol amount were more than 10 times higher in the Bezostaja 1 cultivar, which points to the remarkably larger GSH-GSSG pool. If the relative protein contents of the experimental groups were also taken into consideration, in a general manner, high drought stress caused this value to increase with respect to control groups, as stated in the literature [23]. For the Bezostaja-1 cultivar, the percentage of this incremantal change is about 24%, with values of 137.40 mg/g fresh weight and 170.20 mg/g fresh weight at control and 20% PEG applied groups, respectively. While the Tosunbey control group had an average protein content of 99.60 mg/g fresh weight, the highest drought stress applied group had a value of 128.40 mg/g fresh weight. It should be noted that the overall protein content of the susceptible Bezostaja-1 cultivar is about 35% higher than that of Tosunbey ($p<0.005$).

The calculated proline concentrations showed an interesting change at the uttermost drought application, i.e., this value peaked at the 20% PEG group for both cultivars. While the values of control and 5-10% PEG applications of all experimental groups were in the range of 1-3 mM/g fresh weight, they climbed to 19.11 mM/g fresh weight for the Tosunbey and 49.28 mM/g fresh weight for the Bezostaja-1. The evaluation of those final values also reveals the fact that the Bezostaja-1 cultivar answers the most serious water deficit with nearly 2.5 times higher proline production and accumulation with respect to the tolerant Tosunbey cultivar.

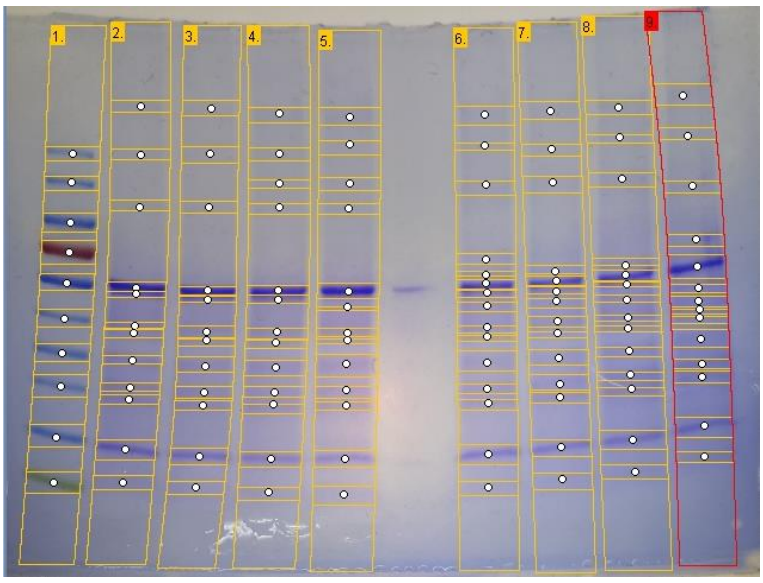

Figure 6. SDS-PAGE gel image of experimental groups. 1: Protein MW marker (from top to bottom: 180 kDa, 130 kDa, 95 kDa, 72 kDa, 55 kDa, 43 kDa, 34 kDa, 26 kDa, 17 kDa, 10 kDa), 2-5: Tosunbey samples (control, 5% PEG, 10% PEG, 20% PEG, respectively), 6-9: Bezostaja-1 samples: (control, 5% PEG, 10% PEG, 20% PEG, respectively).

Homogenates obtained from experimental groups of two cultivars were run with a wide range protein molecular weight (MA) marker in 4-20% gradient gels to which they were added in equal amounts and their profiles were observed by CCB staining (Figure 6). PyElph 1.14 software was submitted for clustering the outcomes of PEG applications on protein band profiles of those two bread wheat cultivars and for generating related phylograms.

Phylogenetic tree and matrix values formed by UPGMA analysis clearly revealed that 10% and 20% PEG treatments for both species resulted in different protein clustering than control and 5% PEG-6000

treatments, i.e., low drought pressure (Figure 7-A). This difference is particularly evident in the Bezostaja 1. In addition to UPGMA, the "neighbor joining" cluster shows that high drought (10% PEG and above) application in the Tosunbey variety creates quite different changes in protein profiles; however, it also reveals, more prominently, that the water deficit groups of Bezostaja-1 (10-20% PEG) reveal a different clustering than any other sample, even including Tosunbey (Figure 7B).

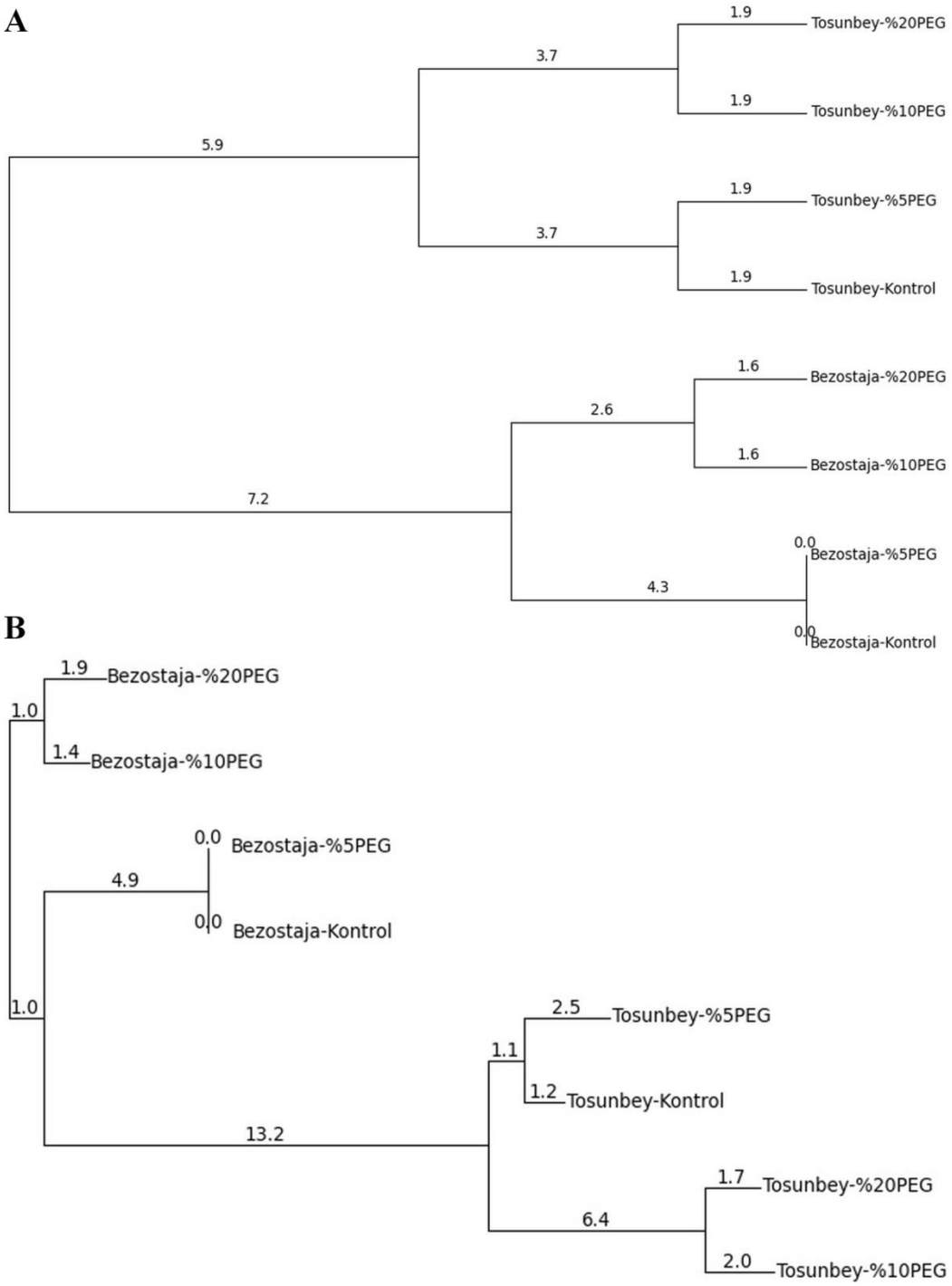

Figure 7. Phylogenetic tree representation of (A) UPGMA and (B) neighbor joining protein cluster analysis results of Tosunbey and Bezostaja-1 sample groups.

DISCUSSION

The evaluation of some basic physiological parameters [24] with biochemical markers including glutathione S-transferases [25], which are important parts of the antioxidant metabolism of plants, is a common and effective way of understanding the underlying mechanism of drought-tolerance. In the present study, two wheat cultivars reported as resistant (Tosunbey) and intolerant (Bezostaja 1) were grown under

the same conditions and exposed to drought stress in PEG-6000 solutions. The osmotic potential created by increasing concentrations of PEG-6000 material, which is not absorbed by plant cells and is non-toxic to tissues [26], has an exponential variation character [27]. This indicates that the drought stress applied in the current study was also strengthened exponentially from 5% PEG to 20% PEG, so that the results were evaluated considering this relationship.

Many studies aimed at uncovering the physiological responses of wheat cultivars to drought stress [28–30] reported lower RWC and length values; and, those parameters showed similar patterns of change in the current study with respect to increasing water deficit. RWC has been used as a main selection criterion for drought resistance in winter wheat [31]; so, with lower rate of decrease (<%20) in this parameter by incresing water scarcity, Tosunbey could be reported as relatively resistant with respect to other cultivar. Proline may act as an osmoprotectant [32], a signal for plant defense mechanisms [33] and even as an antioxidant [34]. Additionally, proline accumulation, which might be considered as an indicator that has been used for a long time but is still popular in assessing drought, cold, and salt stress [35], was used as a tool in the effective evaluation of the response of cultivars. Both cultivars had distinctly raised proline concentrations at high drought condition, therewithal, the Bezostaja-1 cultivar showed a much higher level and variation in the value of proline concentration than the Tosunbey demonstrating its metabolism struggling severe stress.

The levels of leaf chlorophyll content could be used as indicators of the photosynthetic capacity of subjected wheat cultivars; so, the changes in those values are worthwhile to calculate in assessing the success of survivors under heavy drought stress. Drought stress reduces total chlorophyll content and flueroscence in general [36–38]. However, for the early-response of wheat seedlings, there are reports of elevation of this parameter [39,40] possibly related to upregulation in differentially expressed genes coding for some proteins of the chlorophyll metabolism pathway [41]. An increase in chlorophyll-a and chloropyll-b content and stomatal conductance in wheat leaves might improve the efficiency of the light harvesting complex and overall carbon use [42]. The integrity of the photosynthetic apparatus might still be preserved in the early stages of drought application, and the degradation of chlorophyll biosynthesis and down-regulation of related enzymes could take some time. The abundance and prevalence of chloroplasts and the increase in total carotenoids were determined in drought resistant barley varieties [43]. Carotenoids have well-established functions in harvesting light in the paths of photosynthesis, but they also work as quenchers of triplet chlorophyll and singlet oxygen [44]. With the elevation of ROS, an incline in the carotenoid metabolism is highly probable.

There are studies on changes in GST gene levels or protein amounts in wheat cultivars in water deficit conditions; however, there are very few reports on changes in activities. Moreover, no study could be found on the GSTT1 isozyme in wheat. However, GST enzymes are important members of the resistance mechanism activated by plants against many types of abiotic stress, including drought. They play an important role in the removal of ROS created by environmental pressure. It has been reported that industrial plants in which GST genes are over-expressed by transgenic methods provide a great advantage against drought pressure [45]. Both varieties increased their total GST activities at varying rates in extremely dry conditions. The noteworthy point in these measurements is that total GST enzyme activity decreased in 5% PEG applications in both varieties, compared to the control; and, similarly, it increased in 10% PEG and even more in 20% PEG applications in both varieties. In addition, higher specific activity values of GSTs in the Bezostaja-1, which is a susceptible variety, were calculated in all experimental groups, compared to the resistant Tosunbey cultivar. As associated with all these measurements, there is a decrease in the total amount of thiol, which is used as a measure for the size of the GSH pool, inversely with increasing drought. Other studies [46–48] also found a decrease in total GSH-GSSG in both drought-tolerant and sensitive wheat cultivars. In short, the GSH pool decreased in applications where the total GST activity increased. Both the elevated need for the GSH substrate and especially the increased need for antioxidants explain these changes. Moreover, the total GSH pool in sensitive Bezostaja, with higher total GST activity, was found to be more than 10 times larger than that of Tosunbey. This was most probably because of aroused oxidative stress in the Bezostaja-1 cultivar with severely decreased water content. In the selection of varieties that would be advantageous in terms of surviving and maintaining product value in short-term but intense drought periods that will be experienced more frequently in the second half of this century, the preference of those that could maintain their GST activity and those with a larger and more stable GSH pool would be beneficial and important.

GSTT1 activities decreased in both cultivars due to increasing drought. It has been reported that this enzyme is involved in toxin catabolism and there is down-regulation in genetic expression level under drought stress [49]. In the current study, supporting these results, GSTT1 specific activity values decreased inversely with increasing water deficit. More comprehensive studies are needed on the reasons for such a change.

After the SDS-PAGE procedures were applied using the Tosunbey and the Bezostaja-1 sample groups and containing equal amounts of proteins at all lanes, protein cluster analyses were carried out. The aim of UPGMA analysis is to understand to what extent drought pressure causes a change in protein profiles of the same species and how this change creates a pattern of variation between different PEG applications. More marginal changes in protein patterns of wheat varieties may be expected to be observed if they are physiologically unavoidably severely affected by drought. For both varieties, UPGMA phylograms revealed that the protein profiles elicited by moderate to high drought treatments (10% PEG and 20% PEG) were statistically different from those observed in control and low (5% PEG) drought stress groups, as predicted at the beginning of the study. Another point to be emphasized was that the protein profiles of the Bezostaja-1 variety as a result of moderate and high drought applications were different from both the control and 5% PEG applications of the same cultivar and the whole sample groups of the other cultivar. In other words, the protein profiles of the Bezostaja-1 control and 5% PEG samples showed greater similarity to those of Tosunbey's samples than the profiles of the medium and high drought-treated samples of the same cultivar. This might be an indication of how the intolerant Bezostaja-1 cultivar had to change its protein profile obligatorily to avoid medium and high drought. However, more extensive experiments are required to be able to make definitive inferences.

CONCLUSION

Wheat cultivars show different responses to drought stress in a variety of physiological and biochemical ways. Both the cultivars behaved as expected in water deficit conditions; i.e., they elevated osmoprotectant levels, alarmed antioxidant mechanisms and changed their overall protein profile to adapt and survive under stress. Unlike some previously reported studies, the amounts of photosynthetic pigments and, most likely, the photosynthetic rate have increased as the early response, supposably due to the need for energy to keep the defense mechanism working and to make the other necessary preparations. The drought-susceptible Bezostaja-1 cultivar had higher proline and relative protein content, total thiol, and total GST specific activity values than the resistant Tosunbey cultivar, which may explain why the Bezostaja-1 is a more drought-sensitive cultivar and suffers more than Tosunbey under the same water stress conditions. UPGMA and neighbor-joining based analyses supported that Bezostaja-1 exhibited a more dramatic and significant change in the overall protein profile than the other, which could be argued to be associated with an irremediable resistance to water scarcity. In this case, it could be argued that in addition to popular parameters such as relative protein content and proline amount, comparisons of total thiol content, total GST activity and changes in the protein bands might be used as useful parameters in the selection of drought-resistant cultivars at early developmental stages. Further research involving more cultivars with varying resistance capacities would be needed to clearly demonstrate the contribution of biochemical and physiological parameters to the selection of the most drought-resistant cultivars and those that can provide the highest yield under current conditions.

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REFERENCES

1. Bi H, Kovalchuk N, Langridge P, Tricker PJ, Lopato S, Borisjuk N. The impact of drought on wheat leaf cuticle properties. BMC Plant Biol. 2017;17(1):1–13.
2. Trnka M, Feng S, Semenov MA, Olesen JE, Kersebaum KC, Rötter RP, et al. Mitigation efforts will not fully alleviate the increase in water scarcity occurrence probability in wheat-producing areas. Sci Adv. 2019;5(9).
3. Carrão H, Naumann G, Barbosa P. Mapping global patterns of drought risk: An empirical framework based on sub-national estimates of hazard, exposure and vulnerability. Glob Environ Chang. 2016;39:108–24.
4. Langridge P, Reynolds MP. Genomic tools to assist breeding for drought tolerance. Curr Opin Biotechnol. 2015;32:130–5.
5. Łukasik I, Wołoch A, Sytykiewicz H, Sprawka I, Goławska S. Changes in the content of thiol compounds and the activity of glutathione s-transferase in maize seedlings in response to a rose-grass aphid infestation. PLoS One. 2019;14(8).
6. Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, et al. Glutathione in plants: an integrated overview. Plant Cell Environ. 2012;35(2):454–84.

7. Vaseva II, Grigorova BS, Simova-Stoilova LP, Demirevska KN, Feller U. Abscisic acid and late embryogenesis abundant protein profile changes in winter wheat under progressive drought stress. Plant Biol (Stuttg). 2010;12(5):698–707.
8. Chaves MM, Maroco JP, Pereira JS. Understanding plant responses to drought - from genes to the whole plant. Funct Plant Biol. 2003;30(3):239–64.
9. Cheng L, Wang Y, He Q, Li H, Zhang X, Zhang F. Comparative proteomics illustrates the complexity of drought resistance mechanisms in two wheat (Triticum aestivum L.) cultivars under dehydration and rehydration. BMC Plant Biol. 2016;16(1):1–23.
10. Ozturk A. Drought Resistance in Bread Wheat Genotypes. Turkish J Agric For. 1998;23(EK5):1237–48.
11. CIMMYT and GRIS Datasets of Tosunbey [Internet]. [cited 2022 Feb 18]. Available from: http://wheatatlas.org/varieties/detail/28906
12. CIMMYT and GRIS Datasets of Bezostaja-1. [cited 2022 Feb 18]. Available from: http://wheatatlas.org/varieties/detail/22919
13. Smart RE, Bingham GE. Rapid Estimates of Relative Water Content. Plant Physiol. 1974;53(2):258–60.
14. Lichtenthaler HK, Buschmann C. Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. Curr Protoc Food Anal Chem. 2001;1(1):F4.3.1-F4.3.8.
15. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil. 1973;39(1):205–7.
16. Sedlak J, Lindsay RH. Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968;25(C):192–205.
17. Yilmaz C, Iscan M. Glutathione S-Transferase activities and glutathione levels in needles of drought stressed Pinus brutia Ten. trees. Turkish J Biochem. 2014;39(2):238–43.
18. Habig WH, Pabst MJ, Jakoby WB. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem. 1974;249(22):7130–9.
19. Yilmaz C, Bulus H, Oguztuzun S, Cihan M, Fidan C. The activities of GST isozymes in stomach tissues of female obese patients. Turkish J Biochem. 2020;45(6):883–9.
20. Pavel AB, Vasile CI. PyElph - a software tool for gel images analysis and phylogenetics. BMC Bioinformatics. 2012;13(1).
21. Pascal S, Scalla R. Purification and characterization of a safener-induced glutathione S-transferase from wheat (Triticum aestivum). Physiol Plant. 1999;106(1):17–27.
22. Hao Y, Xu S, Lyu Z, Wang H, Kong L, Sun S. Comparative Analysis of the Glutathione S-Transferase Gene Family of Four Triticeae Species and Transcriptome Analysis of GST Genes in Common Wheat Responding to Salt Stress. Int J Genomics. 2021;2021:6289174.
23. Nagy-Réder D, Birinyi Z, Rakszegi M, Békés F, Gell G. The Effect of Abiotic Stresses on the Protein Composition of Four Hungarian Wheat Varieties. Plants (Basel, Switzerland). 2021;11(1).
24. Geravandi M, Farshadfar E, Kahrizi D. Evaluation of some physiological traits as indicators of drought tolerance in bread wheat genotypes. Russ J Plant Physiol. 2011;58(1):69–75.
25. Qi YC, Liu WQ, Qiu LY, Zhang SM, Ma L, Zhang H. Overexpression of glutathione S-transferase gene increases salt tolerance of arabidopsis. Russ J Plant Physiol. 2010;57(2):233–40.
26. Meher, Shivakrishna P, Ashok Reddy K, Manohar Rao D. Effect of PEG-6000 imposed drought stress on RNA content, relative water content (RWC), and chlorophyll content in peanut leaves and roots. Saudi J Biol Sci. 2018;25(2):285–9.
27. Williams J, Shaykewich CF. An Evaluation of Polyethylene Glycol (P.E.G.) 6000 and P.E.G. 20,000 in the Osmotic Control of Soil Water Matric Potential. Can. J. Soil Sci. 2011;49(3):397–401.
28. Sharma V, Kumar A, Chaudhary A, Mishra A, Rawat S, Basavaraj YB, et al. Response of Wheat Genotypes to Drought Stress Stimulated by PEG. Stresses. 2022;2(1):26–51.
29. Qaseem MF, Qureshi R, Shaheen H. Effects of Pre-Anthesis Drought, Heat and Their Combination on the Growth, Yield and Physiology of diverse Wheat (Triticum aestivum L.) Genotypes Varying in Sensitivity to Heat and drought stress. Sci Rep. 2019;9(1).
30. Chowdhury MK, Hasan MA, Bahadur MM, Islam MR, Hakim MA, Iqbal MA, et al. Evaluation of Drought Tolerance of Some Wheat (Triticum aestivum L.) Genotypes through Phenology, Growth, and Physiological Indices. Agron. 2021;11(9):1792.
31. Schonfeld MA, Johnson RC, Carver BF, Mornhinweg DW. Water Relations in Winter Wheat as Drought Resistance Indicators. Crop Sci. 1988;28(3):526–31.
32. Hare PD, Cress WA, Van Staden J. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ. 1998;21(6):535–53.
33. Chun SC, Paramasivan M, Chandrasekaran M. Proline Accumulation Influenced by Osmotic Stress in Arbuscular Mycorrhizal Symbiotic Plants. Front Microbiol. 2018;9:2525.
34. Reddy AR, Chaitanya KV, Vivekanandan M. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol. 2004;161(11):1189–202.
35. Mwadzingeni L, Shimelis H, Tesfay S, Tsilo TJ. Screening of bread wheat genotypes for drought tolerance using phenotypic and proline analyses. Front Plant Sci. 2016;7(AUG2016):1276.

36. Chen D, Wang S, Cao B, Cao D, Leng G, Li H, et al. Genotypic variation in growth and physiological response to drought stress and re-watering reveals the critical role of recovery in drought adaptation in maize seedlings. Front Plant Sci. 2016;6(JAN2016):1241.
37. Rehman SU, Bilal M, Rana RM, Tahir MN, Shah MKN, Ayalew H, et al. Cell membrane stability and chlorophyll content variation in wheat (Triticum aestivum) genotypes under conditions of heat and drought. Crop Pasture Sci. 2016;67(7):712–8.
38. Yasir TA, Wasaya A, Hussain M, Ijaz M, Farooq M, Farooq O, et al. Evaluation of physiological markers for assessing drought tolerance and yield potential in bread wheat. Physiol Mol Biol Plants. 2019;25(5):1163.
39. Nikolaeva MK, Maevskaya SN, Shugaev AG, Bukhov NG. Effect of drought on chlorophyll content and antioxidant enzyme activities in leaves of three wheat cultivars varying in productivity. Russ J Plant Physiol. 2010;57(1):87–95.
40. Terletskaya N. Growth and photosynthetic reactions of different species of wheat seedlings under drought and salt stress. Period Biol. 2017;119:37–45.
41. Zeng R, Chen T, Wang X, Cao J, Li X, Xu X, et al. Physiological and Expressional Regulation on Photosynthesis, Starch and Sucrose Metabolism Response to Waterlogging Stress in Peanut. Front Plant Sci. 2021;12:778.
42. Wang X, Huang M, Zhou Q, Cai J, Dai T, Cao W, et al. Physiological and proteomic mechanisms of waterlogging priming improves tolerance to waterlogging stress in wheat (Triticum aestivum L.). Environ Exp Bot. 2016;132:175–82.
43. Filek M, Łabanowska M, Kurdziel M, Wesełucha-Birczyńska A, Bednarska-Kozakiewicz E. Structural and biochemical response of chloroplasts in tolerant and sensitive barley genotypes to drought stress. J Plant Physiol. 2016;207:61–72.
44. Havaux M. Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci. 1998;3(4):147–51.
45. Kumar S, Trivedi PK. Glutathione S-Transferases: Role in Combating Abiotic Stresses Including Arsenic Detoxification in Plants. Front Plant Sci. 2018;9.
46. Simova-Stoilova L, Demirevska K, Petrova T, Tsenov N, Feller U. Antioxidative protection in wheat varieties under severe recoverable drought at seedling stage. Plant, Soil Environ. 2008;54(12):529–36.
47. Loggini B, Navari-Izzo F, Izzo R. Has Glutathione a Key Role in the Resistance to Oxidative Stress in Durum Wheat? Phyt - Ann Rei Bot. 1997;37:151–6.
48. Chen KM, Gong HJ, Chen GC, Wang SM, Zhang CL. Gradual Drought Under Field Conditions Influences the Glutathione Metabolism, Redox Balance and Energy Supply in Spring Wheat. J Plant Growth Regul. 2004;23(1):20–8.
49. Liao W, Li S, Lu C, Peng M. Tau GSTs involved in regulation of leaf abscission by comparison the gene profiling of MeGSTs in various abscission-promoting treatments in cassava abscission zones. BMC Genet. 2018;19(1).