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Research Article

Memory of wheat to repeated Heat Stress during pre-anthesis could be responsible for improved tolerance

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Abstract

Risk imposed by high temperatures (HT) to the quality and yield of cereals, requires evaluation of naturally resistant resources, and finding of methods to improve it. In this research, were analyzed the tolerance to HT of 19 winter wheat cultivars (*Triticum aestivum* L.) in use in Albania, and the possible memory gained to the repeated stress, before anthesis. Biometric (root, shoot, leafs length), physiological (fine root cells death, Relative Water Content-RWC), and biochemical parameters (*chla*, *chlb*, carotenoids and xantophylls, and total carbohydrates) were measured, and the impact of a short shock (SS) at 42°C/2hrs versus a longer treatment (LT) at 38-35°C/24hrs on pigment synthesis, and on the expression of rubisco activase (*Rca1*) coding gene were investigated. A classification system was built to describe the tolerance to HT, and cultivars were grouped via UPGMA, and PCoA. Results show that SS impacted pigment synthesis more than LT, while expression of *Rca1* was cultivar-specific; In a group of 19 cultivars under two treatments (T1, T2) at 30°C, the vulnerable: moderately tolerant: tolerant were 4: 11: 5, and 3:9:7, respectively. Values were cultivar-specific for single parameters, yet a general trend was evident for some. Improved tolerance to repeated stress was described as gained stress memory.

Keywords: biochemical synthesis, gene expression, primed stress memory, hierarchical clustering.

1. Introduction

Continuous increase in heat waves have contributed to a 0,5°C increase in mean global temperature (Mishra et al, 2021), which have a severe impact on crops growth, yield and product quality as revealed by mathematical modelling (Semenov and Shewry, 2011; Mishra et al, 2021; Zhao et al, 2022). However, the way wheat responds to HT could be varied due to difference in thermotolerance nature of cultivars (Mishra et al, 2018; Mishra et al, 2021). In general, plants do survive and grow in HT thanks to mechanisms classified as: Avoidance and Tolerance (Mishra et al, 2021), or as Avoidance, Escape and Tolerance (Poudel and Poudel, 2020). The first includes various morphological adaptations, expression of genes encoding proteins or accumulation of stress-associated proteins, while the tolerance as described by Mishra et al (2021) involves alterations in expression of genes encoding ion transporters, transcription factors or enzymes involved in osmoprotectants, and antioxidant molecule biosynthesis. The same concept described by Poudel (2020) involves the antioxidant defense, generation of Heat Shock Proteins (HSPs), and Stay Green (SG), with the last (SG) concept associated to genotypes maintenance of photosynthesis and grain filling in HS condition through late expression of senescence related genes. It is clear that understanding the wheat responses to HT is a challenge, while the role of multiomics approaches for this purpose, has not been well documented (Mishra et al, 2021). Wheat productivity is reported to be reduced remarkably due to harmful effect of HT in growth process (Poudel et al, 2020). Thus, the pre-anthesis HT is reported to retard physiological processes such as the pollen viability, seed formation, embryo development (Khan et al, 2020), reduces the crop total duration by causing early flowering and shortening the grain filling period (Mohan et al, 2017; Lamaoni, 2018; Pandey et al, 2022), deactivates the Rubisco enzyme and rubisco activase (Poudel et al, 2020), inhibits the chlorophyll fluorescence and mitochondrial respiration (Iqbal, 2017), reduces the rate of assimilate translocation, and causes a premature leaf senescence (Poudel et al, 2020). Considering the morphological features, the leaf appearance rate, leaf elongation rates, leaf-elongation duration (Liu et al, 2011), number of leaves (Sharma, 2015), root growth (Chauhan et al, 2011), number of roots, root length and root diameter (Iqbal, 2017) are among the well documented characteristics impacted by HT, along with biochemical processes such as the oxidative stress, during which plants highly produce ROS, which interrupt the cell function by their negative actions on lipid, protein and DNA. In this overall context of physiological, biochemical, morphological and agronomical characteristics impacted by HT, the building of thermo-tolerance strategies becomes a necessity for the sustainability of crop yield (Khan et al, 2021). It is already reported that plants can acquire heat stress tolerance (HST) through priming, which establishes stress memory during mild or severe transient heat stress (Wang et al, 2014; Serrano et al, 2019). The impact of heat priming during early vegetative stages (before anthesis) (Wang et al, 2014; Khanzada et al, 2021; Wang et al, 2016), during the stem-elongation stage, booting and anthesis (Fan et al, 2018) on the post-anthesis phases resulted in

higher grain yield under a subsequent high temperature stress (HTS), while during grain filling was pronounced differently in plants primed at the booting stage than in those primed at the stem-elongation or anthesis stage (Fan et al, 2018). Also, a differentiated tolerance displayed by winter wheat plants was evidenced after low heat priming (LP) compared to moderate heat priming (MP), and effects of heat priming applied to the first generation on tolerance of the successive generation to post-anthesis HT were reported (Wang et al, 2016). Proteome analysis indicated that the proteins involved in photosynthesis, energy production, and protein destination and storage were up-regulated in the primed versus non-primed plants. Meanwhile, the metabolomic analysis shows altered energy pathways, and the presence of so-called crosstalk between carbohydrate metabolism and tyrosine metabolism (Serrano et al, 2019). In this overall context, the present study aims to evaluate the tolerance of nineteen winter wheat cultivars in use in Albania, to repeated HT during pre-anthesis. Their selection along with evaluation of the stress response mechanisms related to maintaining photosynthesis rate, biomass growth, water maintenance, and carbohydrate metabolism were assessed in this respect, while previous works of the group had addressed the impact of herbicide treatments (Bacu et al, 2024; 2021; Kokojka et al, 2021), salinity (Bacu et al, 2020a; 2020b), and drought (Ibro et al, 2019).

2. Materials and methods

2.1 Plant material and germination. Seeds of 19 cultivars of winter wheat (*Triticum aestivum* L.) were planted in plastic pots in soil with the following content: organic soil material (95%), and the rest limestone fertilizer, clay, perfit, nitrogen, phosphorus, and potassium (NPK) fertilizers, and other secondary materials, pH value: 5.5, salt content 1.2 g/l potassium chloride. After planting, seeds were allowed to germinate and grow under normal lighting conditions and temperatures ranging from 15°C to 22°C (control conditions), while being periodically watered with tap water for three weeks.

2.2 HT treatment. Two experiments were performed:

- The first aimed to evaluate the impact of short versus prolonged HT treatment on the photosynthetic pigments content, and on the *Rca1* gene expression. For these, ten wheat cultivars (Suba, Artika, Komolica, Isja, Nogal, Dajti, Viktoria, Tombozo, Frederiku, UBT2), were planted in two parallel pots, kept at control conditions (22 +/- 2°C), and allowed to germinate and grow for 4 weeks. After that, half of pots (one per each cultivar) named as the Stressed Group were subjected to the following heat treatments: a short treatment (ST) with a gradual increase up to 42°C for 2hrs; back for 2 days at control conditions; and a long treatment (LT) for 24hrs at 38°C/35°C (day/night regime); Pigments were extracted and measured after each treatment following 2.3.3, and *Rca1* gene expression was controlled after the total leaf RNA was extracted according to Ribaudo (2001). The specific RT-PCR for *Rca1* was performed using the primer pair:Rca1F:5'-

GCTTCTGCTTTCTCATCCAC-3';Rca1R:5'-TGGTCATCGGAGATGTCGTA-3' following Degen (2020). Master–Mix was prepared according to the instructions of the Enhanced Avian HS-RT-PCR (SIGMA) in a single-step reaction, and the RT-PCR products (expected fragment of 224bp) were evaluated in agarose gels 1.2% in TAE, and photographed using the UV Transilluminator VWR Genosmart.

- The second experiment aimed to investigate the possible HT stress memory at wheat plants exposed to two subsequent HT (30°C) treatments (T1 and T2) during pre-anthesis. For this, three weeks old plantlets were exposed at 30°C for 1hr, then kept in control conditions for 48hrs, and subsequently exposed to a second HT treatment of 30°C for 1hr.

2.3 Evaluated Parameters. For the purpose of the second experiment (2.2.b) the following parameters were evaluated:

2.3.1 Morphometric traits (root, stem and leave lengths) were measured manually 7 days after T1 and T2, from five parallel samples (plants) per cultivar.

2.3.2 Relative Water Content (RWC) was measured following (Annuziata et al, 2017) 48hrs after each treatment; For this roots from five parallel samples (plants) per cultivar were cleaned by brushing and immersing in water, dried in tissue paper to remove the moisture, and immediately weighed to obtain the fresh weight. Samples were reassessed after being washed in double-distilled water for 24hrs at 4°C in the dark, and after being dried at 68°C for 48hrs. The relative water content (RWC) was calculated as: RWC = (root fresh weight - root dry weight) / (root turgor weight - root dry weight) x 100

2.3.3 Leaf pigment content was evaluated according to (Hiscox & Israelstam, 1979) 48hrs after T1 and T2; DMSO (dimethyl sulfoxide) was used to extract photosynthetic pigments from leaves of 10 parallel plants per cultivar. Spectrophotometer calibration was performed with pure DMSO and measurements were performed at wavelengths of 470 nm, 645 nm, and 663 nm to see light absorption by chlorophyll a, chlorophyll b, and carotenoids + xanthophylls. The amounts of pigments were calculated using Arnon's (1949) equations.

2.3.4 Total leaf carbohydrates were determined by standard anthrone method (Yemm and Willis, 1954) 48hrs after T1 and T2.

2.3.5 Fine root cell mortality was determined 72hrs after T1 and T2 by counting live cells based on DAPI staining of the fine-root cell DNA, and visualizing via inverted fluorescence microscope (OPTICA) equipped with an Optikam PRO6 Digital Camera. For this, root systems were carefully washed and fine roots (<2mm in diameter) were separated from coarse roots (>2mm in diameter) by careful brushing, then cut into segments not longer than 2mm and stained with DAPI (0, 1μg/μL). Five root segments were examined from each sample with counting of alive cells repeated in 5 different

square areas of the same dimensions (250000 px^2) for each root segment sample, and images were processed using the *optikaproview* software.

2.3.6 Statistical analysis

Past 4,3 software via ANOVA *single-factor* and ANOVA *two-factors with replication* were used to statistically analyse differences among cultivars for HT treatments, and the data were used to produce graphs accordingly.

Dendrogramma of similarity of cultivars based on the response to HT was built using the Hierarchical Clustering, Algorithm UPGMA, via Similarity Index Bray-Curtis of software Past 4,3. The PCoA analysis of cultivars similarity distribution was performed using the PCoA Scatter Plot of software Past 4. In order to understand the trend of the parameters for the whole group of cultivars under study, a generalized schematic presentation of the impact of HT on each single-parameter was built as a Box Plot (Standard error and Standard deviation were considered) via software Past 4.3.

3. Results & discussions

3.1 Impact of short-HT on pigments synthesis

Six out of 10 cultivars (Fig 1) synthesized smaller quantity of total chlorophylls and carotenoids after the short-HS with the amount of *chla* and *chlb* varied in a cultivar-specific manner. Their inability to respond to the stress by synthesizing more carotenoids and xantophylls, which are well-known as metabolites involved in the stress response mechanisms, as well as the lower amount of chlorophyll pigments directly related to the photosynthesis rate, was considered as a lower tolerance (cultivars 1, 2, 4, 5, 6, 9, 10) to HT compared to the rest of cultivars.

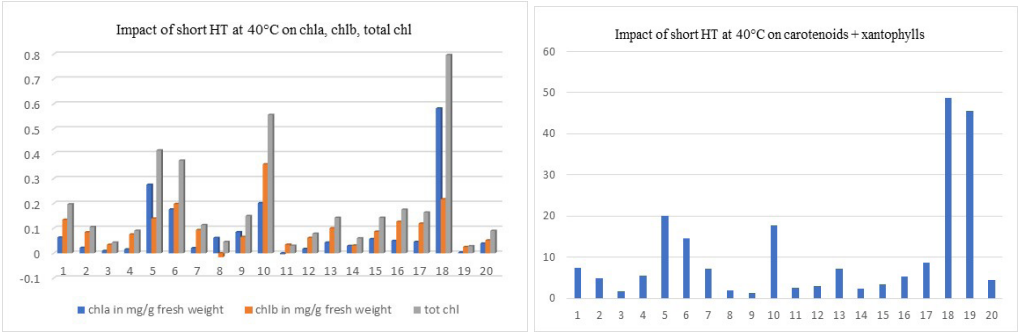


Figure 1. Comparison of the impact of short HT treatment on pigments synthesis (chla, chlb, carotenoids and xantophylls) of different wheat cultivars (1. Suba, 2. Artika, 3. Komolica, 4. Isja, 5. Nogal, 6. Dajti, 7. Viktoria, 8. Toborzo, 9. Frenetiku, 10. UBT-2) during vegetative phase before anthesis. Samples from left to right: Cv 1-10 Control plants; Cv 11-20 Plants under short-HS; From the upper to the lower graph:

A. Variation of chla, chlb, and total chlorophylls (in mg/g of leaves fresh weight). B. Variation of carotenoids and xantophylls.

3. 2 Impact of long- HT on pigments synthesis

Three out of ten cultivars have synthesized less chlorophyll pigments and less carotenoids plus xantophylls after the LT (Fig 2). The comparison of the quantity of carotenoids of stressed plants after ST and LT, shows that the second is higher, proving this way a better response to the stress

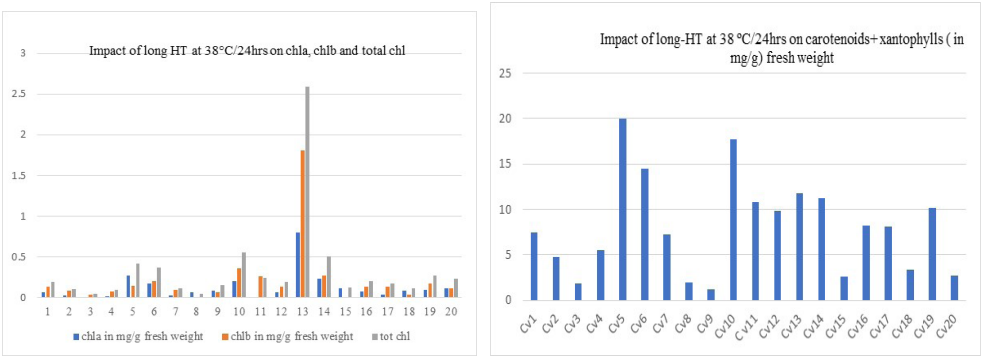


Figure 2. Comparison of the impact of long-HT treatment at photosynthetic pigments of wheat cultivars (1. Suba, 2. Artika, 3. Komolica, 4. Isja, 5. Nogal, 6. Dajti, 7. Viktoria, 8. Toborzo, 9. Frenetiku, 10. UBT-2) before anthesis. From left to right: A. Variation of *chla*, *chlb*, and total chlorophylls. B. Variation of carotenoids and xantophylls. Samples from left to right: Cv 1-10 Control plants; Cv 11-20 Plants under long-HS;

3.3 Expression of *RCa1* after ST versus LT

As seen (Fig 3) the response to the two modes of heat treatment is cultivar-specific with *Rca1* being either less (1, 5, 6, 7) or more expressed (3, 4, 8, 9). Based on previous reports Calvin Cycle (CC) is more sensitive to thermal stress than electron transport by PSII, with the main site of inhibition the activation of rubisco by the rubisco activase (Law and Crafts-Brandner, 1999).

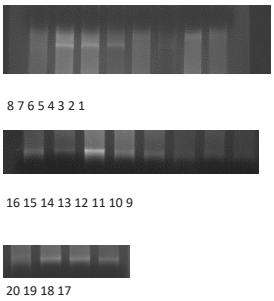
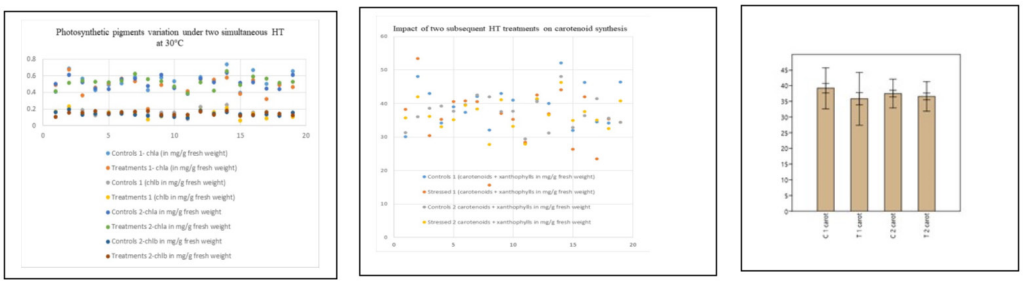


Figure 3.
Compared expression of Rubisco activase (RCa1) at plants under short heat shock at 42°C/2hrs versus longer HT at 38ºC/24 hours.
From 1-10 wheat plants under short HT;
From 11-20 wheat plants under longer HT.
Cultivars: 1. Suba, 2. Artika, 3. Komolica, 4. Isja, 5. Nogal, 6. Dajti, 7. Viktoria, 8. Toborzo, 9. Frenetik, 10. UBT-2.

With increasing temperatures, it seems that Rubisco activase becomes less capable to maintain the active state of Rubisco after it is denatured. Our results show that some of the cultivars have very low amount of expression after the heat shock at 42°C (3, 8, 9, 10), compared to LT indicating this way their susceptibility.

3.4 Possible HT memory as revealed by:

3.4.1 Pigments synthesis



a **b** **c**

Figure 3. Impact on pigment synthesis of two subsequent HT applications at wheat cultivars.

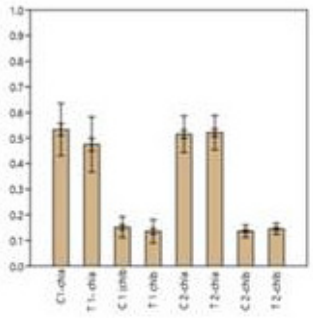
a. Upper-left graphic: *chla* and *chlb* after the two simultaneous HT at 30ºC.

b. Upper-right graphic: carotenoids and xantophylls after the two simultaneous HT at 30ºC.

c. Lower-left graphic: Generalized schematic presentation of the impact of HT at chlorophyll a and b synthesis. From left to right: C1chla; T1Chla; C1chlb; T1chlb; C2chla; T2chla; C2chlb; T2chlb. d. Lower-right graph: Generalized schematic presentation of the impact of HT at carotenoids and xantophylls synthesis.

From left to right: C1carot; T1carot; C2carot; T2carot. Graphs 3c and 3d were built as Box Plot using the Standard error and Standard deviation of values for each cultivar via software Past 4.3.

Samples from 1-19: Cultivars Lushnja, Laci, Toborzo, Sirtaki, Korce, Ciari, Urma, Urma-Adelajde, MV-Toborzo, Eli, Krajlica, Vittoria, Artillo, Frenetik, Vittoria-P3, UBT-1, Dajti,

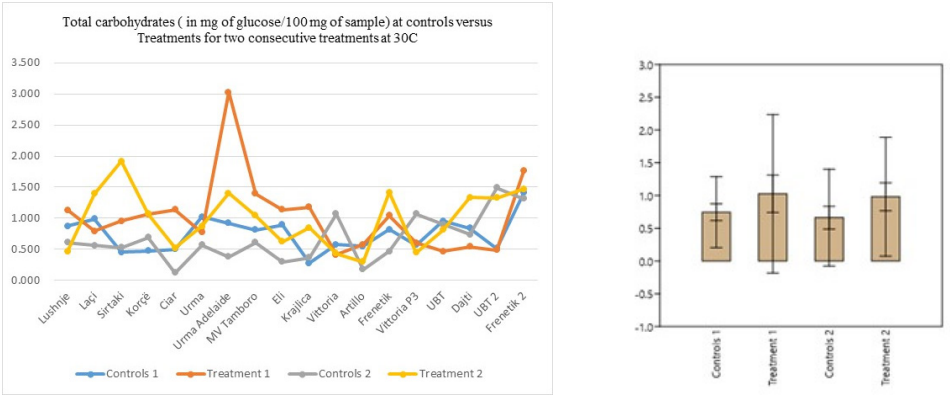


d

UBT-2, Frenetik-2.
Eleven out of nineteen cultivars synthesize less *chla* and *chlb* after T1, compared to five out of nineteen after T2. Twelve out of nineteen cultivars synthesize less carotenoids and xantophylls after T2 compared to T1 (Fig 3.c-d). Considering the fact that the HTs were subsequent, results can be explained as memory from the first HS through developing mechanisms to protect pigment synthesis, and this way to keep the photosynthesis rate un-impacted. The same could be said for the oxidative stress management via carotenoids and xantophylls, which after the second HS were found in lower concentrations (Fig 3.c-d).

3.4.2 Total leaf carbohydrates

Among the reported responses of wheat to HT are the ones, which consider maintaining plant hydration and enhancing carbohydrate remobilization to grains, and evidence of the interplay between plant water relations and carbohydrate metabolism (Habti, 2020).



a b

Figure 4. Impact on carbohydrate synthesis of two subsequent HT applications at wheat cultivars.
a. Comparison of mean total carbohydrate amounts for stressed and control plants for each cultivar.
b. Generalized schematic presentation of the impact of HT at total carbohydrate content, built as Box Plot (Standard error and Standard deviation were considered) via software Past 4.3.
Samples from 1-19: Cultivars Lushnja, Laci, Toborzo, Sirtaki, Korce, Ciari, Urma, Urma-Adelajde, MV-Toborzo, Eli, Krajlica, Vittoria, Artillo, Frenetik, Vittoria-P3, UBT-1, Dajti, UBT-2, Frenetik-2.

On the other hand, carbohydrate synthesis and transport are closely related to water movements in plants, with the soluble ones also playing an important role during drought by acting as compatible osmolytes to maintain cell turgor and favorable plant water status, thereby sustaining biological processes and soil water uptake (Blum, 2017; Habti, 2020). According to (Padam, 2020) there is an increase in carbohydrate

remobilization from stem to developing grains during pre-anthesis HS, which helps to recover the effect on grain starch content in post-anthesis HS. Our results show that 12 out of 19 cultivars have higher amount of carbohydrates after T1, and 13 out 19 after T2. The difference in the quantity of carbohydrates between controls and treated plants is cultivar-specific, but the trend is clearly toward the increased production of carbohydrates for more than 2/3 of the cultivars (Fig 4.a-b).

3.4.3 Fine root cell mortality

Fine root dynamics describe the production, growth, mortality, and decomposition of fine roots, (Wang et al, 2019), while, it is already reported that wheat cultivar replacement can alter root morphology and anatomy, thereby improving grain yield and water use efficiency (Xiaofei et al, 2024). In this study the mortality of fine-root cells under HS was considered as a parameter to be used to evaluate the tolerance displayed by wheat cultivars (Fig 6).

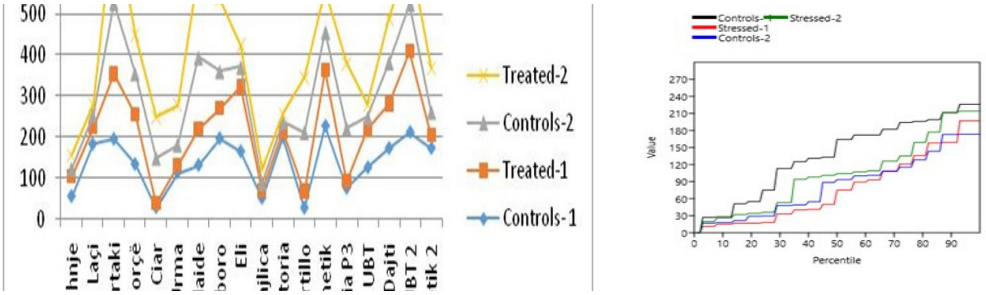


Figure 5. Comparison of the mean average number of alive fine root cells at stressed versus control plants exposed to two subsequent HT treatments. Counting was based on DAPI staining, and visualization via fluorescence microscopy. On the right: the generalized trend of values as produced by software Past 4.0. Samples from 1-19: Cultivars Lushnja, Laci, Toborzo, Sirtaki, Korce, Ciari, Urma, Urma-Adelajde, MV-Toborzo, Eli, Krajlica, Vittoria, Artillo, Frenetik, Vittoria-P3, UBT-1, Dajti, UBT-2, Frenetik-2.

Results show that only 17 out of 19 cultivars had lower number of alive fine root cells after T1, and 4 out 19 after T2 (Fig 5) from which only 4 (cultivars Lushnja, Korca, Ciar and Vittoria) had lower number of alive fine root cells after T1 and T2 compared to controls. The last were considered less tolerant, meanwhile the fact that 13 out of 19 cultivars were not impacted by T2 was interpreted as gained stress memory.

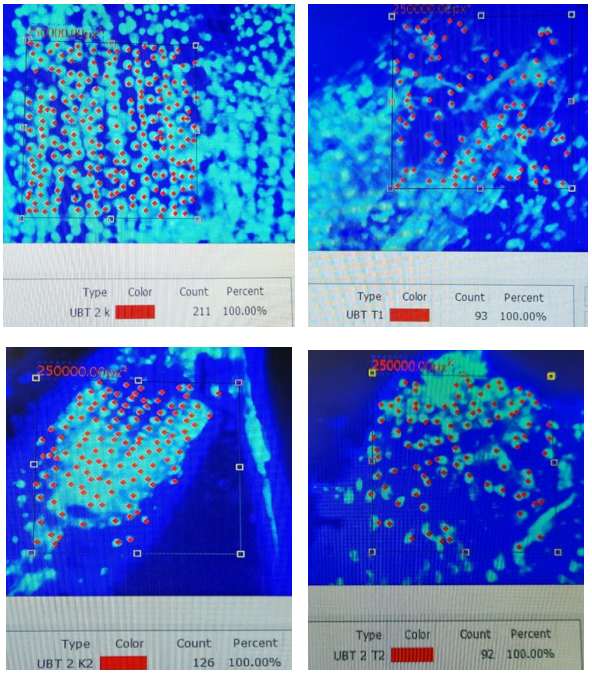


Figure 6. Determining the impact of two subsequent HT at 30°C on live/dead cells at fine roots of wheat based on staining of the root cell DNA with DAPI, and visualizing via inverted fluorescence microscope
Pictures of the upper part from left to right display: control plants for Cultivar UBT-2 (UBT-2-K), and stressed plants after first treatment (UBT-2-T1);
Pictures of the lower part from left to right display: control plants for Cultivar UBT-2 (UBT-2-K2), and stressed plants after first treatment (UBT-2-T2);

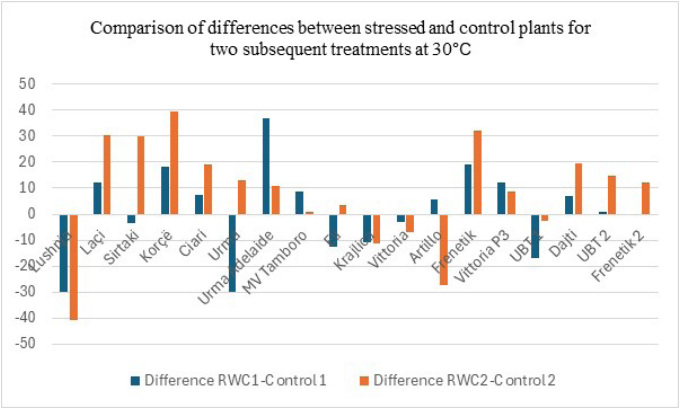


Figure 7. Determining the impact of two subsequent HT at 30°C on RWC.
The graph displays values of $RWC_{stressed}$ - $RWC_{control}$ per each cultivar.

3.4.4 Relative Water Content-RWC

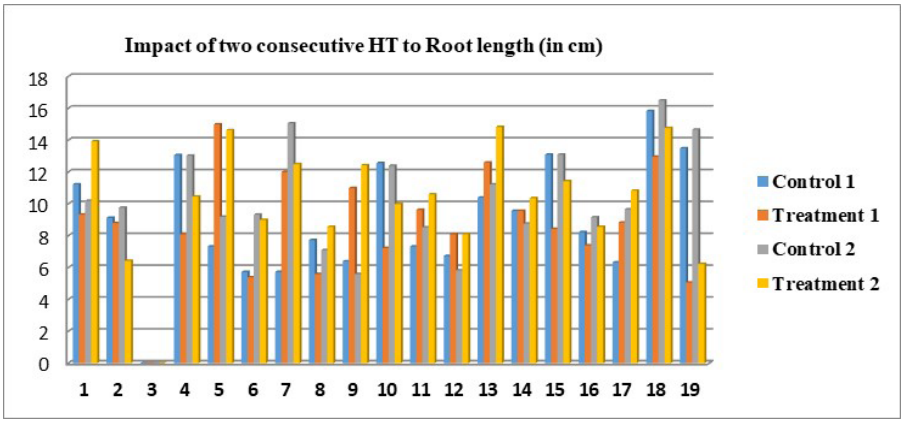
HS affects the water content in plants through cell dehydration due to reduction in osmotic potential (Ahmad et al, 2010; Poudel et al, 2020), leaf relative water content, stomatal conductance, and transpiration (Sharma et al, 2019; Poudel et al, 2020).

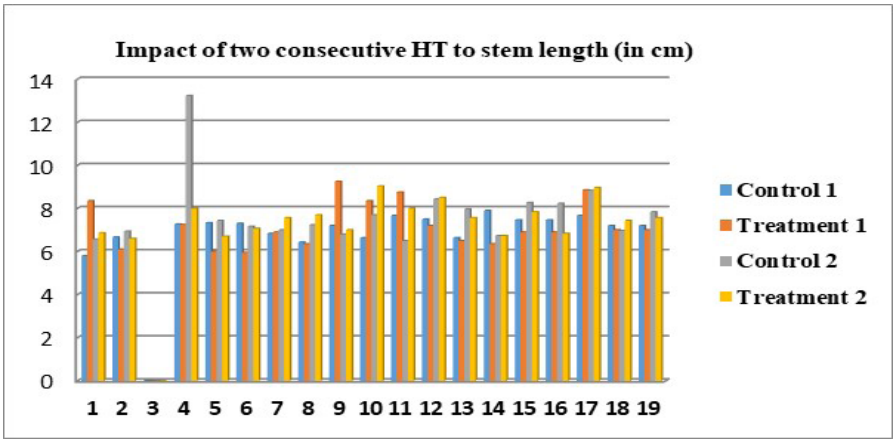
During the vegetative stage, transpiration drives biomass accumulation, which in turn results in high water use during grain filling when water is available (Habti, 2020).

In our study, for 10 out of 19 cultivars (T1), and 13 out of 19 cultivars (T2) the difference of RWC between stressed and control plants was positive. Among them, 10/19 have a higher water content after T2 compared to T1, suggesting this way a possible stress memory mechanism to water uptake and retention in wheat leaves. For 7 cultivars RWC was lowered after T1, and for 4 of them even more after T2 compared to T1. The cultivar-specific response for this parameter is in accordance with previous reports which state that although all vegetative and reproductive stages are affected by HS to some extent, yet the stage of plant development also shows varied degree of vulnerability to high temperature (Wahid et al, 2007; Iqbal, 2017). Biomass and water use are linearly related (De Wit, 1958), and mutually dependent during the plant's life cycle (Habti, 2020), and the maintaining transpiration following heat stress confirms the strong relationship between plant transpiration and yield (Sinclair, 2005; Habti, 2020). In accordance to the above, cultivars Lushnja, Krajlica, Vittoria, Artillo, and UBT-1, which have lowered RWCs after T1 and T2, were considered as less tolerant to HS compared to the rest.

3.4.5 Morphometric parameters

As compared to other growth processes, root growth has a very narrow range of optimum temperature (Prasad et al, 2006) with the decreased number of roots, root length and root diameter as the manifestations of heat stress. Significant differences were found for biometric parameters between the 19 soft wheat cultivars related to their response to high temperatures (Fig 9). Among them, 6, 8, 15, 16 and 19 stand out as very sensitive; while 2, 12, 14, 16, 18 could be considered as relatively sensitive. On the other hand, cultivars 7, 11 and 17 appear to be more tolerant. Most of the 19 wheat cultivars, after T2, reawaken the resistance acquired after T1. Typical for this reaction are 8, 6 and 14 showing increased growth for the three evaluated organs; roots, stems and leaves. While cultivars 1, 12, 18, 2, 4, 5, 9, 10 and 13 manifest increased growth for two or one of the evaluated organs after T2. Cv. 7, 11 and 17, in addition to being tolerant after T1, after T2 react with a significant increase in the length of their roots, stems and leaves. Contrary to the above, cv 15, 19 and 16 do not themselves appear very sensitive to high temperatures, but do not reawaken any reaction (adaptation) after T2. High temperatures are generally involved in regulation of leaf appearance rates and leaf elongation rates along with decreasing leaf-elongation duration (Roberts, 1987). Heat stress is reported to have resulted in significant increase in number of leaves, particularly during the arrested reproductive development stage and without any decrease in leaf photosynthetic rate (Bos et al, 2000).





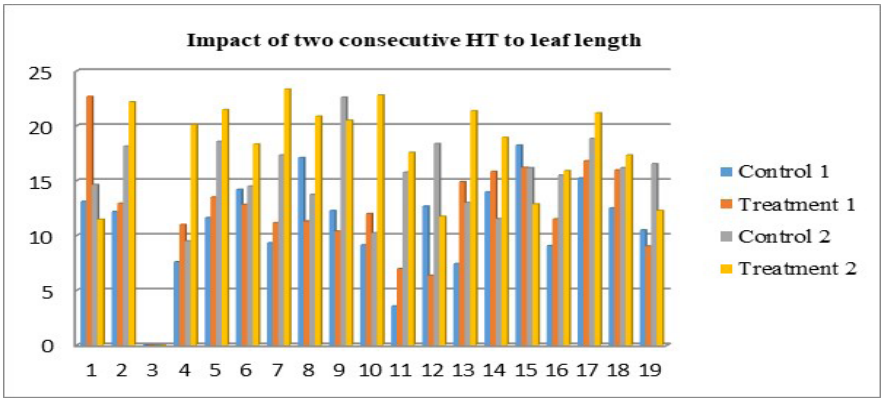


Figure 8. Root, shoot and leaf elongation after T1 and T2. Samples from 1-19: Cultivars Lushnja, Laci, Toborzo, Sirtaki, Korce, Ciari, Urma, Urma-Adelajde, MV-Toborzo,

Eli, Krajlica, Vittoria, Artillo, Frenetik, Vittoria-P3, UBT-1, Dajti, UBT-2, Frenetik-2.

As evidenced from this study (Fig 8), cultivars displayed a cultivar-specific mode of response for morphometric parameters measured. They were included in the data set used to build the dendrogram of similarity (Fig 10), and in the PCoA analysis (Fig 11) of the similarities displayed by cultivars, as well as to build Tables 1 and 2, which represent the thermo-tolerance classification system database and score results (Fig 9).

a. Root Length

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	494.6187	18	27.47882	15.32113	1.34E-11	1.907346
Within Groups	62.77333	35	1.793524			
Total	557.392	53				

B. Stem Length

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.05426	18	0.780792	1.775296	0.071605	1.907346
Within Groups	15.39333	35	0.43981			
Total	29.44759	53				

C. Leaf Length

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	36.93139	18	2.051744	3.129623	0.011395	2.256671
Within Groups	11.145	17	0.655588			
Total	48.07639	35				

Figure 9. Description of the results of the ANOVA statistical analysis for morphometric parameters.

3.5 Comparison of the response of cultivars to HT & Classification of their tolerance level

TABLE 1. Summary of the responses of different wheat cultivars to the T1 (30ºC/1hr), as revealed by morphometric, biochemical and physiological data.

Response to First HT treatment									
Cultivars	RWC	Photosynthetic pigments			Total carbohydrates	Fine roots cell death	Morphometrics		Total score (in *)
		chla	chlb	Carotenoids + xantophylls			Root length	Shoot Length	
Lushnja	↑**	↑**	↑**	↑**	↑**	↑	↓	↑**	12
Laçi	↓	↓	↑**	↑**	↓	↓**	↓	→*	7
Toborzo-1	↓	↓	↓	↓	↑**	↓**	↑**	→*	7
Sirtaki	↑**	↑**	↑**	↑**	↑**	↓**	↓	→*	13
Korçë	↓	↑**	→*	↑**	↑**	↑	↑**	↓	9
Ciari	↓	↑**	→*	↑**	↑**	↓**	→*	↓	10
Urma	↑**	↓	↓	↓	↓	↓**	↑**	↑**	8
Urma- Adelaide	↓	↓	↓	↓	↑**	↓**	↓	↑**	6
MV Toborzo-2	↓	↓	↓	↓	↑**	↓**	↑**	→*	7
Eli	↑**	↓	↓	↓	↑**	↑	↓	↑**	6
Krajlica	↑**	→*	↑**	→*	↑**	↓**	↑**	↑**	14
Vittoria	↑**	→*	↓	→*	↓	↓**	↑**	↑**	10
Artillo	↓	→*	→*	↓	↑**	↓**	↑**	↓	7
Frenetic-1	↓	↓	↓	↓	↑**	↓**	↑**	→*	7
Vittoria (P3)	↓	↓	↓	↓	↑**	↓**	↓	↓	4
UBT 1	↑**	↓	↓	↓	↓	↑	↓	→*	3
Dajti	↓	↓	↓	↓	↓	↓**	↑**	↑**	6
UBT 2	↓	→*	→*	→*	↓	↑	↓	→*	4
Frenetic 2	→*	↓	↓	→*	↑*	↓**	↓	↓	5

Leend:

↑ values increased; ↓ values decreased; → values unchanged;

* Score when parameter is unchanged

** Score when parameter is changed positively toward the tolerant version

TABLE 2. Summary of the responses of different wheat cultivars to the T2 (30ºC/1hr), as revealed by morphometric, biochemical and physiological data.

Response to Second HT Treatment									
		Photosynthetic pigments					Morphometrics		Total score (in *)
Cultivars	RWC	chla	chlb	Carotenoids +xantophylls	Total carbohydrates	Fine roots cell death	Root length	Shoot Length	
Lushnja	↑**	↓	↓	↑**	↓	↓**	↑**	→*	9
Laçi	↓	↓	↓	↑**	↑**	↓**	↓	→*	7
Toborzo-1	↓	↑**	↑**	↓	↑**	↓**	↑**	↑**	12
Sirtaki	↓	↑**	↑**	↓	↑**	↓**	↓	↓	8
Korçë	↓	↑**	↑**	↑**	↑**	↓**	↑**	↓	12
Ciari	↓	↓	↑**	↑**	↑**	↓**	→*	→*	10
Urma	↓	↑**	↑**	↓	↑**	↓**	↓	↑**	10
Urma Adelaide	↓	↑**	↑**	↓	↑**	↓**	↑**	→*	11
MV Toborzo-2	↓	↓	↑**	→*	↑**	↓**	↑**	↑**	11
Eli	↓	↑**	↑**	↓	↑**	↑	↓	↑**	8
Krajlica	↑**	→*	↑**	↓	↑**	→*	↑**	↑**	12
Vittoria	↑**	↓	↓	↑**	↓	↓**	↑**	→*	9
Artillo	↑**	↓	↓	↑**	↑**	↑	↑**	↓	8
Frenetic-1	↓	↑**	↑**	↓	↑**	↓**	↑**	→*	11
Vittoria (P3)	↓	↓	↓	↑**	↓	↓**	↓	↓	4
UBT 1	↑**	↑**	↑**	↑**	↓	↓**	↓	↓	10
Dajti	↓	↑**	↑**	↓	↑**	↓**	↑**	↑**	12
UBT 2	↓	↑**	↑**	↓	↓	↓	↓	→*	5
Frenetic 2	↓	↓	↓	↑**	↑**	↓	↓	→*	5

Legend:

↑ values increased; ↓ values decreased; → values unchanged;

* Score when parameter is unchanged

** Score when parameter is changed positively toward the tolerant

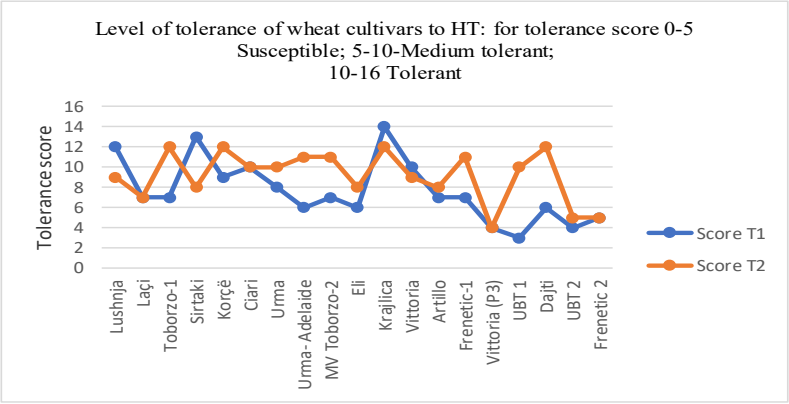


Figure 9. Classification of the tolerance level displayed from 19 cultivars. Data on which the classification system is based were described at Tab1 and Tab2.

As seen (Fig 9) the ratio between *vulnerable* (cultivars that score 0-5): *moderately tolerant* (score 6-10): *tolerant cultivars* (score 11-16) were 4: 11: 5, and 3:9:7 after T1 and T2, respectively, proving an improvement in the overall tolerance of the cultivars, which can be explained as priming effect of the repeated HT. Cultivars Toborzo, Korca, Urma-Adelajde, Krajlica, Frenetik-1, and Dajti displayed the highest level of tolerance to HT in the group.

3.5.1 Level of similarity of different wheat cultivars in response to HT

Five sub-clusters were identified (Fig 9) from which samples 12 dhe 16 (Vittoria and UBT-1) share the highest level of similarity of 95%, and cultivar 19 (Frenetik) has the lowest level of similarity to the rest (70%), in a range of over 87% displayed by all of them in response to repeated HS;

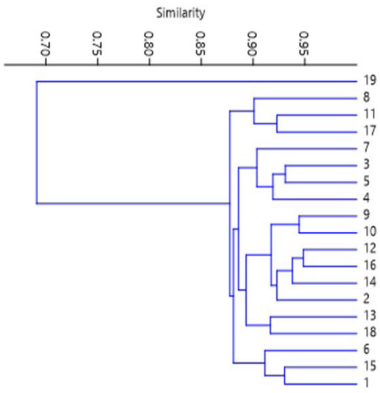


Figure 10. Dendrogramme of the similarity of the response of wheat cultivars towards two subsequent HT at 30ºC (based on photosynthetic pigments, total carbohydrates,

RWC, morphometric parameters). The dendrogramme was prepared using Hierarchical Clustering, Algorithm UPGMA, Similarity Index Bray-Curtis, software Past 4. Samples from 1-19: Cultivars 1. Lushnja, 2. Laci, 3. Toborzo, 4. Sirtaki, 5. Korce, 6. Ciari, 7. Urma, 8. Urma-Adelajde, 9. MV-Toborzo, 10. Eli, 11. Krajlica, 12. Vittoria, 13. Artillo, 14. Frenetik, 15. Vittoria-P3, 16. UBT-1, 17. Dajti, 18. UBT-2, 19. Frenetik-2.

PCoA analysis (Fig 10) was useful to understand the distance in the response of different cultivars, identifying sample 19 as the most distant one, followed by 1, 7, 8, 13, 18, and can therefore be used to select the proper cultivars from a group.

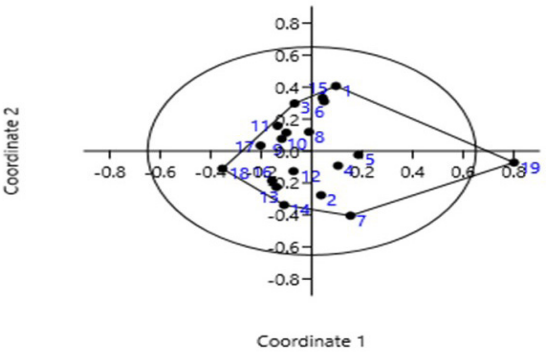


Figure 11. PCoA Analysis of the similarity of the response of different wheat cultivars towards two subsequent HT treatments at 30ºC. PCoA Scatter Plot was built via software Past 4.3.

4. Conclusion

Quality characteristics of importance for the utilization of wheat such as flour protein concentration, milling yield, rheological properties and bread-making properties are influenced by genotype and genotype x environment interactions, and depend upon the intensity, timing and duration of HS. Although all vegetative and reproductive stages are affected by HS to some extent, yet the stage of plant development also shows varied degree of vulnerability to high temperature. In the conditions of climate changes, which have led to an increase in temperature, salinity and drought the discovery of the natural resistance of cereals and finding of methods for the creation of new adapted cultivars constitute an urgent need. This study aimed to evaluate the tolerance of 19 local cultivars of soft wheat (*Triticum aestivum* L.) in use in Albania to high temperature during vegetative development prior to anthesis. To achieve the objective, two experiments were designed:
Experiment A, aimed to evaluate the impact of two modes of HT treatment, short shock (SS) at 42°C/2hrs, and longer treatment (LT) at 38ºC/24hrs, on pigment synthesis (*chla*, *chlb*, carotenoids, xanthophylls), and on the expression of the rubisco activase coding gene (*Rca1*).
Experiment B, during which plants were exposed to two consecutive treatments at 30ºC, was used to evaluate the level of tolerance and the possibility of primed stress memory. For this a classification system was built based on morphometric parameters

(length of root, stem, leaves, number of leaves), biochemical (chla, chlb, carotenoids and xanthophylls, total carbohydrates), and physiological (cell mortality of fine roots, RWC), which made possible the categorization of cultivars into sensitive-moderately tolerant and tolerant, and also the dataset was used to group them (UPGMA similarity, and PCoA analysis), according to similarity. An improvement in the tolerance level of the group was evidenced after T2 compared to T1, as the ratio between vulnerable: moderately tolerant: tolerant cultivars were 4: 11: 5, and 3:9:7, respectively. For single parameters the degree of the variation of responses was cultivar-specific, yet a general trend could be clearly distinguished for most of them (higher RWC, number of alive fine-root cells, and amount of total carbohydrates compared to controls). This brings to discussion the possibility that the modified response to the repeated HT stress applied could be considered as primed stress memory. Thus, after T2 the carotenoids amount was lowered indicating a lower oxidative stress level; the number of alive fine-root cells was higher indicating an improved resistance; total carbohydrates were higher in stressed than control plants; RWC was higher for more than 2/3 of the cultivars. Hierarchical Clustering and PCoA analysis made possible clustering of cultivars and the investigation of the distance on their response to HT, enabling this way an easier process for the selection of more tolerant versus non-tolerant cultivars. Meanwhile, the investigation of the impact of ST compared to a LT showed that the first impacted stronger pigment synthesis, while the expression of *Rca1* was cultivar-specific (lower for four out of ten cultivars). Since pigment synthesis and *Rca1* are strongly related to the maintenance of photosynthesis, with the second being responsible for the activation of rubisco, these can be used to characterize not only the level of tolerance to HT, but also to elucidate the metabolic pathways involved in the response displayed by different cultivars.

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