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# Effects of high temperature during anthesis and grain filling on physiological characteristics of winter wheat cultivars

Wheat physiological response to heat

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**Abstract**

Due to climate change, multiple heat events are expected to be an additional limiting factor that will adversely affect wheat production. The study aimed to analyze the physiological response to heat stress in four winter wheat cultivars at different physiological stages under greenhouse conditions during 2019. Net photosynthetic rate, stomatal conductance, chlorophyll index, maximum quantum efficiency of photosystem II, fructose, glucose and sucrose content, grain yield per plant, grain weight and number of grains per plant were analyzed in wheat cultivars under short periods of heat stress at anthesis and mid-grain filling, and combined stress at anthesis and mid-grain filling. The results of the study indicated that heat stress modified the photosynthesis-related and grain yield-related traits. Moreover, heat stress caused a decrease of sucrose content, while fructose and glucose content increased. Heat stress, had more pronounced effects on the photosynthetic parameters and grain yield during grain filling than during anthesis. A significant variation observed between cultivar responses to the negative impact of heat stress highlighted the fact that cultivars Pobeda and Gladius were more tolerant than Renesansa and Simonida. Different cultivar reactions to heat stress during anthesis and grain filling indicated the need to conduct further studies with wheat cultivars of different origin in order to identify additional sources of tolerance.

**Keywords:** carbohydrate, flowering, grain yield, heat stress, photosynthesis, *Triticum aestivum* L.

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## 1. INTRODUCTION

Heat stress represents a significant abiotic stress influencing crop growth, development and production (Semenov & Shewry, 2010; Yang et al., 2017). According to different scenarios, annual temperatures are expected to increase by 2.0 - 4.9 °C until the end of this century due to climate change (Raftery et al., 2017). Different crop models proposed by Asseng et al. (2014) indicate that global wheat production would decline by 6% per 1°C of temperature increase. Moreover, different climate change scenarios predict a notable temperature increase across the European climate regions, with an additional intensification of heat waves and drought episodes during different wheat development phases (Trnka, Hlavinka, & Semenov, 2015). Observing the main European climate regions, the Pannonian Plain is one of the wheat production regions most vulnerable to the changing climate due to the ongoing increase in the frequency of dry periods and heat waves during anthesis (Trnka et al., 2014). The Pannonian Plain, one of the largest flat basins in Central Europe, is already characterized by frequent drought and heat stress during the period of anthesis and post-anthesis. The predicted temperature increase will be an additional factor which limits wheat production (Olesen et al., 2011).

Heat stress leads to different morphological, anatomical, physiological, and biochemical changes in wheat, influencing grain yield and quality (DuPont & Altenbach, 2003; Barnabás, Jäger, & Fehér, 2008). Leaf photosynthesis is among the physiological processes most affected by increased temperatures (Ristic et al., 2008). Initial decrease in the photosynthetic activity under higher temperatures is caused by the reduced mesophyll conductance and stomata closure (Chaves, Flexas, & Pinheiro, 2009). In addition, the net photosynthetic rate declines due to the changes in the light-dependent and dark reaction (Ashraf et al., 2013). Photosystem II (PSII) is the prominent heat-sensitive component of photosynthesis which limits photochemistry at increased temperatures (Zhou et al., 2017). Changes in PSII are commonly accessed by chlorophyll *a* fluorescence measurements, a quick non-destructive method for stress tolerance analysis (Baker & Rosenqvist, 2004). High temperatures inhibit the activity of sucrose synthase and invertase, restraining sucrose content and metabolism (Dai et al., 2015). Moreover, heat stress reduces chlorophyll biosynthesis and content, leading to early crop maturation (Farooq et al., 2011).

Wheat is the most important crop in Europe, accounting for more than 30% of global wheat grain production (FAOSTAT, 2020). Since wheat is a temperate crop, it is considered heat sensitive. Hence, its prospective yield is considerably reduced under increasing temperatures (Wang et al., 2015). However, in field crops, the influence of increased temperatures varied across wheat development stages (Prasad, Bheemanahalli, & Krishna Jagadish, 2017). The negative effect of heat stress on wheat grain yield is more pronounced during the reproductive period than in vegetative stages (Farooq et al., 2011). Generally, grain yield is determined by grain number and established during pre-anthesis and shortly after anthesis period, while grain weight is determined during the grain-filling period. In small grain cereals, grain yield is more related to the grain number than grain weight (Mirosavljević et al., 2018). Heat stress during the period of pre-anthesis and anthesis strongly limits grain number by influencing ovary development, pollen germination and pollen tube growth (Pradhan et al., 2012). Grain weight is determined by grain filling rate and duration. High temperature during the grain-filling period accelerates crop senescence (Chen et al., 2018), resulting in shorter grain filling. Although grain-filling rate rises by temperature increase, it fails to compensate grain weight reduction due to shorter grain filling period (Prasad et al., 2008).

Information about wheat cultivars response to heat stress effect across different phenological stages is essential for securing wheat production under the predicted climate changes. At present, knowledge about the reaction of bread wheat cultivars to the combined influence of heat stress at anthesis and grain filling is limited. Genotype-dependent responses were reported when comparing single and combined effects of pre-anthesis drought and post-anthesis heat stress on durum wheat genotypes (Liu, Able, & Able, 2019). Winter wheat genotypic differences have been observed previously under a single-day heat shock at flowering stage or early grain set (Talukder et al., 2014) and separately under different temperature levels, heat stress durations at anthesis and grain filling (Liu et al., 2016), while the combined effects at anthesis and grain filling stages were not examined. Since there is a significant genotypic variation among wheat cultivars in the physiological and agronomical traits across the main developmental stages (Vignjevic et al., 2014; Hlaváčová et al., 2018), further analyses of different wheat genepools are necessary to identify tolerant wheat genotypes that could be included in breeding activities. Under field conditions of the Pannonian Plain, winter wheat cultivars are likely to encounter prolonged heat stress during flowering and grain filling. Having recognized the importance of the Pannonian wheat production under unfavorable conditions, the present study aimed to analyze the physiological responses to single and combined heat stress in four winter wheat cultivars in terms of photosynthesis, chlorophyll fluorescence, carbohydrate content and grain yield traits at different physiological stages. Knowledge about the changes of wheat physiological response to heat stress can be used to adjust breeding strategies and improve wheat cultivars adapted to heat-prone environments.

## 2. MATERIALS AND METHODS

### 2.1 Plant materials and treatments

The experiment was conducted at the Department of Food Science, Aarhus University, Aarslev, Denmark. Four wheat cultivars were used in the study, namely 'Renesansa', 'Pobeda' and 'Simonida' (Institute of Field and Vegetable Crops, National Institute of the Republic of Serbia, Serbia), and cultivar 'Gladius' (Australian Grain Technologies, Australia), with previously determined high heat stress tolerance (Fleury et al., 2010). The Serbian cultivars 'Renesansa', 'Pobeda' and 'Simonida' were chosen based on their high yield potential and high bread-making quality. Two seeds were sown per plastic pot (9 cm height, 11 cm diameter) filled with a commercial peat based potting substrate (Pindstrup Færdigblanding 2, PindstrupMosebrug A/S, Ryomgaard, Denmark). In total 25 plants of each cultivar were used for each of the different treatments. The plants were maintained in a greenhouse with a 12/ h photoperiod, combining natural and supplementary light, 48 ± 5% air relative humidity, average temperature of 23.8 °C ± 1.5 °C and ambient $CO_2$. When plants reached the three fully developed leaves stage (Zadoks 13, approximately 14 days after sowing/DAS), seedlings were thinned to one plant per pot and transferred to a cold chamber (4–6 °C, 8 h day length) for 8 weeks for vernalisation. Pots with vernalized seedlings were returned to greenhouse and grown until the anthesis phase (Zadoks 60; Zadoks et al., 1974). Then, 20 uniform plants per cultivar were transferred to the climate chambers at anthesis stage (MB teknik, Brøndby, Denmark). The photoperiod was 14 h with 500 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) provided by LED FL300 Sunlight (Fionia

Lighting, Søndersø, Denmark) with 65% relative humidity and 400 ppm $CO_2$ concentration in the climate chambers. The day length was set from 5:00 to 19:00 in both chambers. Plants were watered with a nutrient solution (190 ppm N; 35 ppm P; 275 ppm K, pH 6.0) three times per day by flooding the bench for approximately 10 min. A 1.5 % w/v sodium bicarbonate in water solution was used to prevent the occurrence of powdery mildew. As a result, there was no fungal infection during the experiment.

The first subset of plants was grown in the climate chamber with 24/16 °C air temperature as control (T1). The responses of the other subsets of wheat cultivars to heat stress were assessed under different developmental stages: (T2) 35/25 °C at anthesis for seven days, (T3) 38/28 °C at mid-grain filling for seven days (14 days after anthesis) and (T4) 35/25 °C at anthesis for seven days and 38/28 °C at mid-grain filling for seven days. After heat stress treatments, the plants were moved back to the greenhouse and grown under previously described conditions to the full ripening stage (DC 92), when the manual harvest was carried out. From each pot, plant spikes were harvested individually. Grain yield per plant (GYP), grain weight (GW) and number of grains per plant (NGP) were calculated after hand thrash of material.



## 2.2 Measurements

Net photosynthetic rate ($P_n$) and stomatal conductance ($g_s$) were measured using a portable open system infrared gas analyser (CIRAS-2, PP Systems, Amesbury, USA) at the seventh stress day (SD7) on the three plants. The flag leaf was placed in a 1.7 cm$^2$ cuvette with a $CO_2$ concentration of 400 ppm, PPFD of 1500 μmol photons m$^{-2}$s$^{-1}$, under the temperature of 24 °C for control, and 35 °C or 38 °C for stress measurements. When the leaf equilibrated in the cuvette conditions, the measurements were recorded every 10 s until $P_n$ and $g_s$ reached a steady state.

Chlorophyll content of the flag leaf was measured using a handheld chlorophyll meter SPAD-502 (Minolta Ltd, Osaka Japan) and referred to as chlorophyll indices (CI). Three random spots from the upper flag leaf side were measured on the five plants and averaged at the first stress day (SD1), the third stress day (SD3), the fifth stress day (SD5) and the seventh stress day (SD7).

Chlorophyll *a* fluorescence was measured on the main stem flag leaves of five plants during the heat treatment periods. The measurements were conducted using a Mini-Pam fluorimeter (Walz Gmbh, Effeltrich, Germany) during afternoon after at least 10 h of light exposure at the (SD1, SD3, SD5 and SD7. Prior to measurements, leaves were dark adapted for at least 30 min with a randomly placed dark leaf clips. Maximum chlorophyll fluorescence ($F_v/F_m$) was measured on the upper leaf surface using a PPFD of 3500 μmol $m^{-2}s^{-1}$ as a saturating flash.

Carbohydrate concentrations were determined in leaf samples taken from the flag leaf of the second stem of three plants at the SD7. Leaf samples were stored in liquid nitrogen at -80 °C until measurements. The samples were then dried, weighed and ground with a steel ball using a mixer mill (MM200, Retsch Inc., Haan, Germany). The extraction of the soluble sugars (glucose, fructose and sucrose) was done first with 80 % ethanol, then 50 % ethanol and 20 % HEPES until the samples turned pale. The supernatants with the soluble sugars were polled and analysed by ion chromatography with a pulsed amperometric detector (PAD) and a gold electrode (Dionex, ICS 3000, Sunnywale, Canada), using 200 mM NaOH as the eluent. To break down the starch into glucose, sterile ultra-pure water was added to the tubes with the leaf pellet, which were then autoclaved for 90 min and mixed with an enzyme buffer solution.

The samples were centrifuged and the glucose units filtered and measured like the soluble sugars.

### 2.3 Statistical analysis

Statistical analysis was carried out using two-way ANOVA performed in INFOSTAT software, while a randomized complete block design with five replications was set up so as to control the heterogeneous effect of temperature and radiation within the greenhouse. Tukey's test ($P < 0.05$) was applied to test the significance among different treatments, cultivars and their interactions at anthesis and mid-grain filling separately for gas exchange, leaf carbohydrate content and grain yield traits. The effect of the treatments on CI and $F_v/F_m$ were analysed using one-way ANOVA for each cultivar separately to show changes in their values during stress treatments duration.

## 3. RESULTS

### 3.1 Gas exchange, chlorophyll content and fluorescence

Figure 1 shows that heat stress effect on $g_s$ and $P_n$ varied across cultivars and treatments. Heat stress at anthesis (T2) significantly increased $g_s$ in all four wheat cultivars as compared with the control plants (Figure 1A). At mid-grain filling (T3), Gladius and Simonida had higher $g_s$ than Renesansa and Pobeda under control conditions (T1). Heat stress decreased $g_s$ significantly in all four wheat cultivars as compared with the control treatment (Figure 1B). Moreover, when multiple heat stress treatments at anthesis and mid-grain filling (T4) were imposed, a drastic decline in $g_s$ was recorded in all cultivars compared with T1 and T3 (Figure 1B).

Figure 1. Stomatal conductance ($g_s$; A and C) and net photosynthesis rate ($P_n$; B and D) in four winter wheat cultivars Renesansa, Pobeda, Simonida and Gladius, grown under control conditions (T1), and seven days during the heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4). Different letters indicate statistically significant differences among treatment and cultivar means tested by two-way ANOVA with Tukey's post hoc test ($P < 0.05$). Values are expressed as the mean ± standard deviation (n = 3).

At anthesis, cultivar Renesansa showed lower $P_n$ than other cultivars under control conditions, while Pobeda showed higher $P_n$ than Gladius and Renesansa under stress treatment (T2) . The $P_n$ of the heat-treated plants at anthesis was significantly lower in Gladius than in the control plants (T1), whereas the differences between Simonida, Pobeda, Renesansa and the control were not significant (Figure 1C). It was observed that the effect of heat stress at mid-grain filling had a more adverse effect than that recorded during the anthesis stage (Figure 1D), since heat stress at mid-grain filling (T3), decreased $P_n$ in all cultivars compared to the plants grown in control conditions (T1). Furthermore, the effect of the multiple heat stress (T4) was the most severe, resulting in the lowest values of $P_n$.

The CI significantly decreased due to the heat stress treatment at both anthesis and mid-grain filling stages (Figure 2). At anthesis, the cultivars showed different response to the increased temperature regarding CI. There was no significant decrease in CI in any of the cultivars on the first day of heat stress (SD1) compared to control conditions. However, the CI significantly decreased in Pobeda from the SD3 onwards, while significant CI reduction was observed in Renesansa, Simonida and Gladius after SD5, and it remained lower over the next days. Finally at SD7, CI reduction varied from 6% (Pobeda) to 18% (Renesansa).

Figure 2. Chlorophyll index in four winter wheat cultivars Renesansa (A and B), Pobeda (C and D), Simonida (E and F) and Gladius (G and H) grown under control conditions (T1), heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4) for four stress days (the first stress day – SD1; the third stress day – SD3; the fifth stress day – SD5; the seventh stress day – SD7). Different letters indicate statistically significant differences among treatment means tested by ANOVA with Tukey's post hoc test ($P < 0.05$) within stress day, separately. Values are expressed as the mean ± standard deviation ($n = 5$).



At the mid-grain filling stage, the effect of heat stress on CI was more pronounced than the heat stress during anthesis period (Figure 2). Compared to the control, heat stress at mid-grain filling (T3) significantly reduced CI in Renesansa at the SD3, while significant decrease was recorded in Pobeda, Simonida and Gladius at the SD5. Across the stress days, reduction in CI increased during the treatment, showing the highest decrease at the SD7. The repeated heat stress at both stages had the most limiting effect on CI in wheat plants. The most significant CI reduction was recorded during the SD1 (except in Simonida). During the stress period, CI decreased almost linearly in all cultivars, with the highest reduction at the SD7, when CI was 80-91% lower in comparison to the plants under control conditions.

Also, the $F_v/F_m$ of the cultivars significantly changed during the heat stress treatments at anthesis and mid-grain filling period (Figure 3). During the T2 treatment, $F_v/F_m$ varied in relation to heat stress days. Compared to the control treatment, Renesansa maintained the $F_v/F_m$ during the 5 days of heat stress, and only at SD7 the value of this parameter decreased significantly. In Pobeda and Gladius, $F_v/F_m$ declined significantly in relationship to the control at SD1, in Simonida at SD5, and remained significantly lower during the following stress days.

Figure 4. Carbohydrate analysis (glucose – A and B fructose – C and D; sucrose – E and F) of the second stem flag leaf taken at the seventh stress day during anthesis (T1 – control anthesis; T2 – the heat stress at anthesis) and at the seventh stress day during mid-grain filling period (T1 – control mid-grain filling; T2 – heat stress mid-grain filling; T3 – combined stress at anthesis and mid-grain filling) in the four winter wheat cultivars Renesansa, Pobeda, Simonida and Gladius. Different letters indicate statistically significant differences among treatment means tested by ANOVA with Tukey's post hoc test ($P < 0.05$). Values are expressed as the mean ± standard deviation ($n = 3$).

Concerning the carbohydrate content, the effect of heat stress at mid-grain filling was more pronounced regarding the carbohydrate content at anthesis. The rising of temperature at mid-grain filling (T3) significantly increased fructose and glucose content because of heat stress compared to the control plants in all cultivars. On the other hand, sucrose content significantly declined in heat stressed plants. In addition, the cultivars responded to heat stress differently regarding sucrose content, as greater reduction occurred in Simonida and Renesansa than in Gladius and Pobeda under T3 and T4 treatments. Thus, Pobeda and Simonida showed higher fructose and glucose content under T4 than Gladius and Renesansa. Furthermore, in Pobeda and Simonida, the highest fructose and glucose content was recorded under conditions of combined stress, in Renesansa fructose and glucose content was higher under T3, while Gladius showed no significant differences in carbohydrates content between T3 and T4.

**3.3 Grain yield traits**

There was a significant difference in GYP potential among the cultivars, where Pobeda showed higher GYP under control, T2 and T3 than other cultivars (Figure 5). The comparisons of GYP across treatments showed a general pattern with the highest value in the control, low to moderate reduction in T2 and T3 conditions and the lowest GYP in the T4. Moreover, the cultivars reacted differently to the stress treatments. Heat stress at anthesis and mid-grain filling resulted in similar GYP decrease in Renesansa, Simonida and Gladius, while Pobeda had a higher GYP decline in T2 conditions compared with T3.

Fig. 5. Grain yield per plant (GYP; A), grain weight (GW; B) and the number of grains per plant (NGP; C) in the four winter wheat cultivars Renesansa, Pobeda, Simonida and Gladius grown under control conditions (T1), seven-day heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4). Different letters indicate statistically significant differences among cultivar and treatment means tested by ANOVA with Tukey's post hoc test ($P < 0.05$). Values are expressed as the mean ± standard deviation ($n = 5$).

Generally, cultivars differed in GW across treatments, and Pobeda and Simonida showed higher GW under T1 and T2. Moreover, differences in GW reduction among the cultivars indicated different sensitivity to heat stress treatments. Heat stress at anthesis did not reduce GW. The highest GW reduction was recorded under heat stress at mid-grain filling and under combined stress. Similar to GW, differences in NGP among the cultivars to heat treatments were observed. Pobeda produced the highest NGP under T1 and T3, while Renesansa was characterized by the lowest NPG under all treatments. Although heat stress at anthesis and mid-grain filling resulted in significant NGP reduction in all cultivars, the effect of T2 was more pronounced. The combined stress treatments resulted in the highest NGP reduction in all cultivars.

## 4. DISCUSSION

Heat stress negatively influences wheat growth during different development stages more than one-half of the total wheat growing area has been experiencing periods of high temperature (Cossani and Reynolds, 2012). Also, the growing conditions in the Pannonian Plain are marked by the frequent occurrence of heat stress during the anthesis and grain filling (Trnka et al., 2014). The influence of heat stress on wheat performance at different phenological stages are usually focused at one specific stage (Hütsch et al., 2019; Schittenhelm et al., 2020), while knowledge about multiple stress combinations is scarce. Unlike many previous studies, we applied heat stress treatments at anthesis and mid-grain filling, and a combined stress treatment at both phenological stages (T4 treatment). This experimental design allowed us to determine the sensitivity of different wheat varieties to the predicted multiple heat waves in the future (Trnka et al., 2014).

Our results revealed that the photosynthesis-related parameters showed different response to heat stress at anthesis and mid-grain filling. Heat stress at anthesis resulted in stomatal conductance ($g_s$) increase in all cultivars, which is probably linked to the increased demands for leaf cooling (Sharma et al., 2015). Besides, there was a decrease in $P_n$,, only significant in the cultivar Gladius (close to the $P_n$ of Renesansa and Simonida recorded during

T2 treatment), which could be directly related to the decrease in $F_v/F_m$ at the SD7 in all cultivars and other non-stomatal effects, such as reduced electron transport (Yan et al., 2013). During mid-grain filling $g_s$ and $P_n$ decreased in all cultivars, most pronounced under conditions of combined heat stress. The $F_v/F_m$ and CI results supported the gas exchange measurements and explained the high reduction in wheat photosynthesis. All cultivars followed a similar trend of changes in gas exchange traits under the applied heat stress treatments.

In small grain cereals, stay green traits are closely related to grain yield traits and improved nitrogen metabolism, showing improvement during the past century due to the breeding activities (Kitonyo et al., 2017). The SPAD readings are commonly used to assess the leaf chlorophyll index and stay green traits (Lopes & Reynolds, 2012; Xiong et al., 2015). In our study, the influence of heat stress on the changes in CI varied across the treatments and the cultivars. The heat stress effect on CI was less pronounced at anthesis than during mid-grain filling, showing only 6-18% reduction in CI after 7 stress days compared to the control. Generally, heat stress damages the thylakoid membrane, resulting in chlorophyll degradation and acceleration of crop senescence rate (Ristic, Bukovnik, & Prasad, 2007). The most severe CI loss was observed during mid-grain filling, especially under the conditions of combined stress. Our results reported different response of cultivars to heat stress at mid-grain filling. The loss of CI was highly induced by heat stress in Renesansa, while Pobeda and Gladius had a slower senescence rate. As previously reported by Mirosavljević et al. (2020), higher crop greenness at mid senescence could be related to the additional increase of photosynthetic activity during the grain filling period, supporting a higher grain yield of crops.

$F_v/F_m$ is often used as an effective indicator of PSII activity and heat stress response or tolerance in different wheat cultivars (Sharma et al., 2015). Heat stress at anthesis had significant influence on $F_v/F_m$, resulting in a decrease of this parameter. In Simonida and Renesansa, significant decrease in $F_v/F_m$ was observed after SD5 and SD7, indicating a delayed effect of high temperature on PSII. On the other hand, heat stress during mid-grain filling had a severe negative effect on $F_v/F_m$, especially under combined stress conditions. $F_v/F_m$ decreased almost linearly in Renesansa and Pobeda under mid-grain filling heat stress, while a significant reduction was delayed to SD5 in Simonida and Gladius. Under combined heat stress conditions, the cultivars had lower $F_v/F_m$ at the SD1 during mid-grain filling, indicating that the plants were not able to restore the $F_v/F_m$ after heat stress at anthesis during the recovery period between the heat stress treatments.

There was a significant variation in carbohydrate content between heat stress treatments. Cultivar ability to produce assimilates during the heat stress is an indicator of heat tolerance (Vignjevic et al., 2015). During anthesis, carbohydrate content mostly remains stable regardless of the heat stress effect. Wheat cultivars could not maintain a high sucrose level in leaf during the heat stress at mid-grain filling and combined heat stress. On the other hand, fructose and glucose content increased due to heat stress at mid-grain filling. There was a significant difference among wheat cultivars regarding fructose and glucose content at mid-grain filling (T3), where Pobeda and Simonida tend to have higher content in comparison to other cultivars. Differences in the carbohydrate content could be used for determination of heat tolerant genotypes as previously reported by Rong et al. (2017), since heat-sensitive genotypes are not able to maintain a high carbohydrate assimilation during the heat stress. On the other hand, higher leaf sugars concentration could be associated with decreased photosynthetic rate due to the feedback control. In addition, there was a close relationship between high sugar content and chlorophyll decrease followed by accelerated crop senescence (van Doorn, 2008). Moreover, Griffiths, Reynolds, & Paul (2020) reported a negative relationship between leaf glucose content and grain yield in wheat.

In our study, heat stress significantly affected GYP. However, the effect on GYP determinants (GW and NGP) varied across the growing stages and cultivars. At anthesis, the influence of heat stress was more related to the NGP reduction, affecting GW at mid-grain filling, while combined heat stress at both stages had the most negative effect on both traits. The reduction of NGP due to heat stress at anthesis varied significantly across the cultivars, with the greatest reduction occurring in Pobeda and Renesansa.At anthesis, heat stress generally decreases grain number per spike due to flower abortion and pollen sterility, influencing final grain yield (Mesihovic et al., 2016). However, in our study, heat stress at anthesis also reduced GW in Renesansa. The grain weight decrease resulting from plant exposure to high temperature at anthesis could be related to lower floret growth rate and carpel size, as previously described by Ugarte, Calderini, & Slafer (2007). Heat stress had a more pronounced effect on GW during mid-grain filling than at anthesis or the control. Heat stress after anthesis mostly affected the efficiency of grain filling, resulting in decreased GW and a significant decrease in the analyzed photosynthesis related parameters ($g_s$, $P_n$, $F_v/F_m$ and CI). Moreover, heat stress during mid-grain filling resulted in significant NGP reduction. The decline in NGP was mainly due to grain abortion during early grain filling, as Tomás et al. (2020) reported. As expected, the highest GYP reduction was reported under combined stress conditions due to the negative effect on both NGP and GW formation. There was a significant variation between cultivar responses to heat stress (Balla et al., 2019). As a result of high GW and NGP, Pobeda had higher GYP potential under control conditions than other cultivars, as well as resulting in higher GYP reduction under heat treatments. Despite the reduction, GW and NGP in Pobeda were generally higher than in the other cultivars under all heat treatments, indicating that Pobeda was able to achieve higher GY under unfavorable conditions. Moreover, Pobeda and Gladius had higher GYP at increased temperature than Renesansa and Simonida, which could both be classified as more heat sensitive. In Pobeda, heat stress tolerance could be more related to retaining higher GW, while in Gladius to maintaining higher NGP during heat stress.

In conclusion, our results showed that higher temperature at anthesis and mid-grain filling influenced the carbohydrate content, as well as affecting the photosynthesis-related and grain yield-related traits. The grain yield reduction due to the heat stress at anthesis was less notable than the heat stress at mid-grain filling and combined stress during both stages. High temperatures at mid-grain filling and combined heat stress had a more negative effect on the photosynthetic related parameters and carbohydrate metabolism, resulting in higher grain weight and grain yield reduction. There was a significant variation in the cultivar response to the negative effects of heat stress, revealing that Pobeda and Gladius are more tolerant than Renesansa and Simonida. Pobeda and Gladius had higher values of $F_v/F_m$ and CI parameters, indicating that the maintenance of the crop photosynthetic activity during heat stress could be a possible strategy for developing more heat tolerant cultivars. Studies should therefore aim to compare wheat cultivars of different origin in order to identify the sources of tolerance to combined heat stress which could be widely used in breeding programs.

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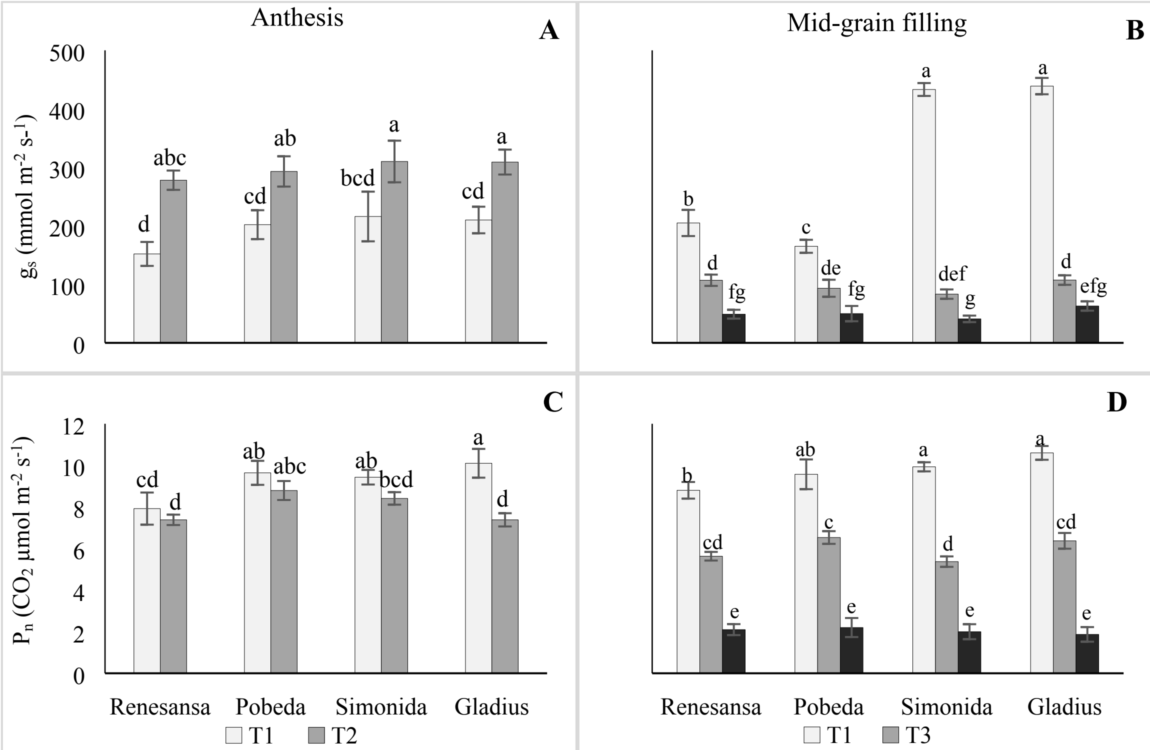


Fig. 1. Stomatal conductance ($g_s$; A and C) and net photosynthesis rate ($P_n$; B and D) in four winter wheat cultivars Renesansa, Pobeda, Simonida and Gladius grown under control conditions (T1), and seven days during the heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4). Different letters indicate statistically significant differences among treatment and cultivar means tested by two-way ANOVA with Tukey's post hoc test ($P < 0.05$). Values are expressed as the mean ± standard deviation ($n = 3$).

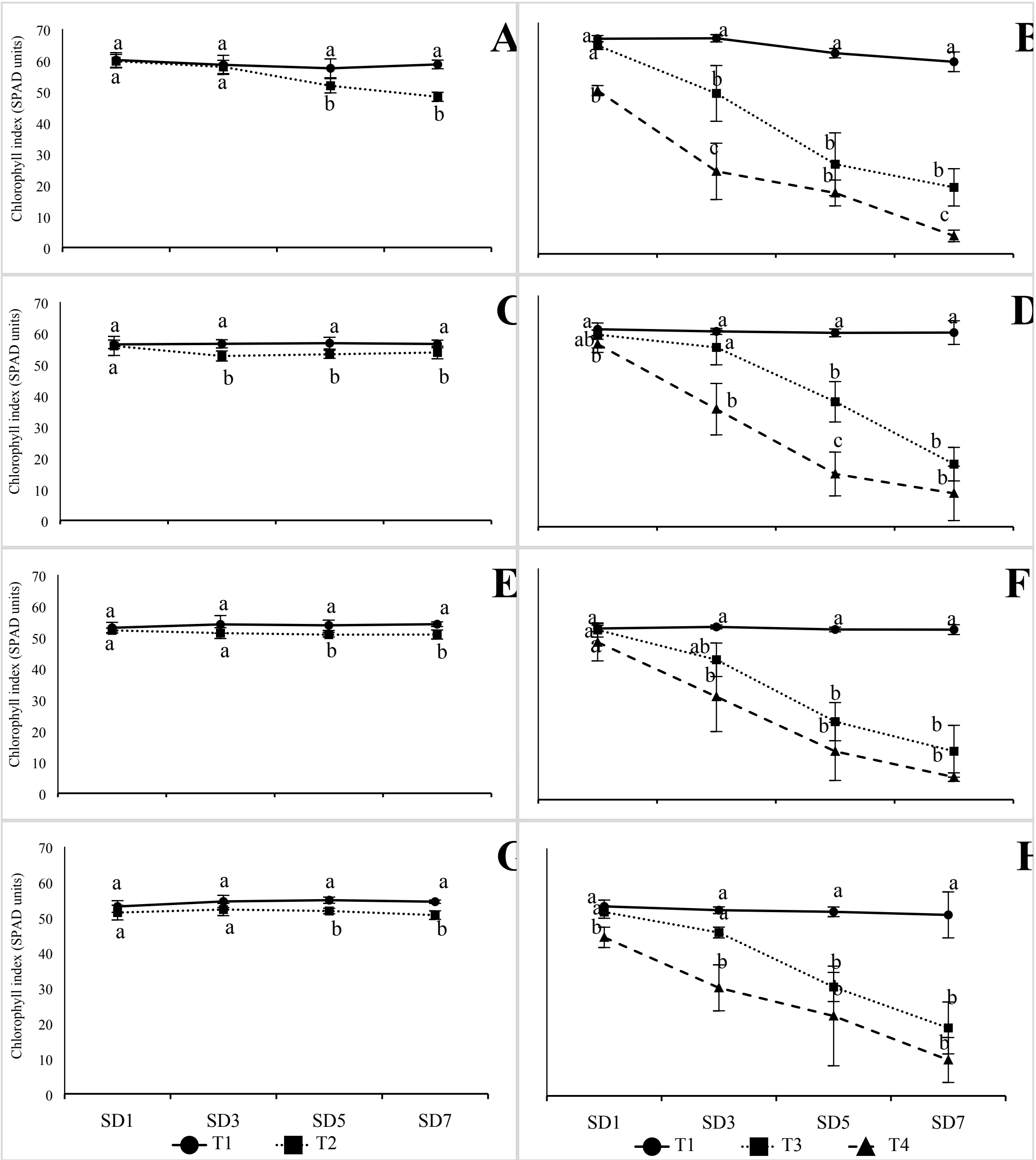


Fig. 2. Chlorophyll index in four winter wheat cultivars Renesansa (A and B), Pobeda (C and D), Simonida (E and F) and Gladius (G and H) grown under control conditions (T1), heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4) for four stress days (the first stress day – SD1; the third stress day –SD3; the fifth stress day – SD5; the seventh stress day – SD7). Different letters indicate statistically significant differences among treatment means tested by ANOVA with Tukey's post hoc test ($P < 0.05$) within stress day, separately. Values are expressed as the mean ± standard deviation ($n = 5$).

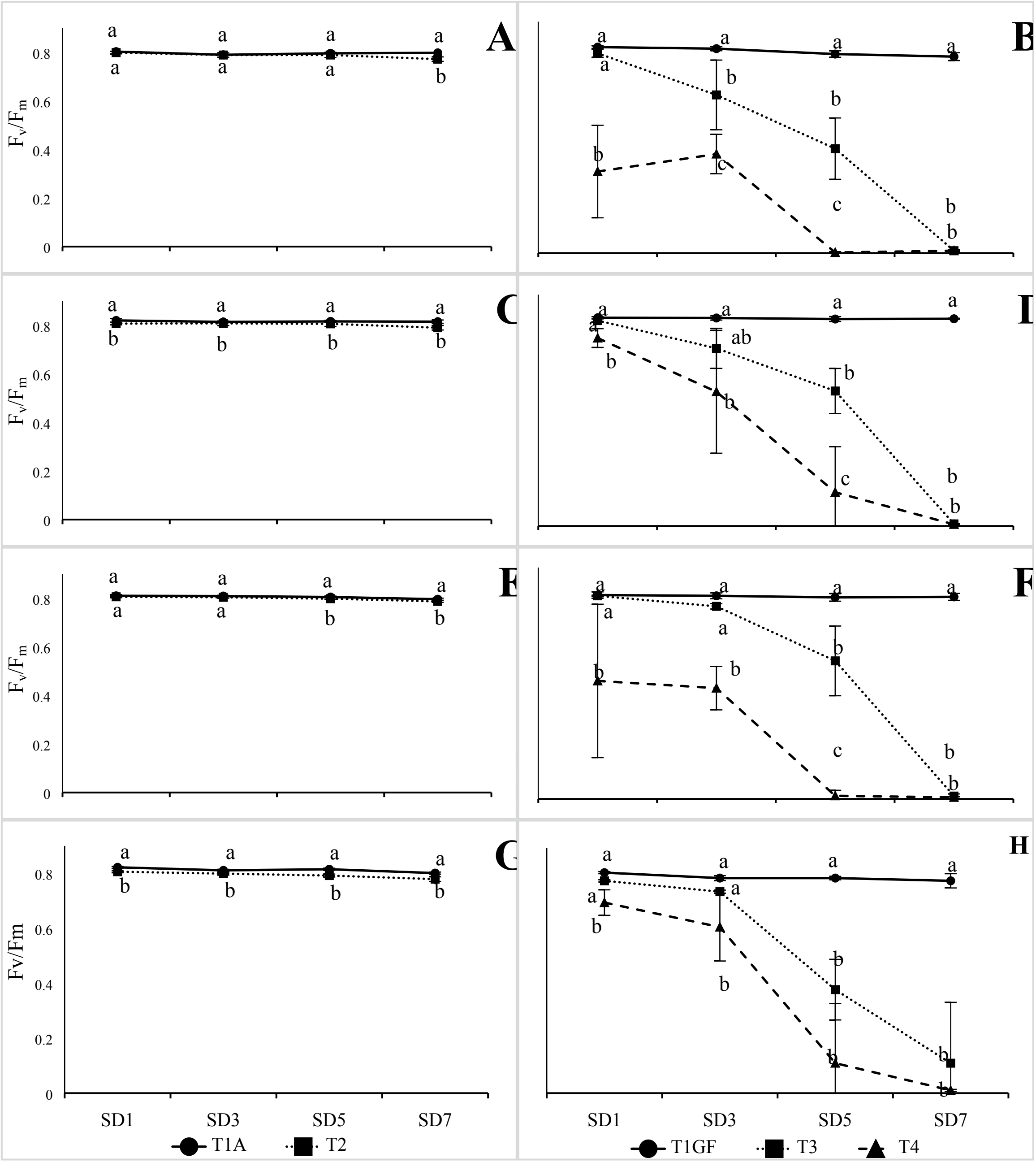


Fig. 3. Maximum quantum efficiency of PSII (Fv/Fm) in four winter wheat cultivars Renesansa (A and B), Pobeda (C and D), Simonida (E and F) and Gladius (G and H) grown under control conditions (T1), heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4) for four stress days (the first stress day – SD1; the third stress day – SD3; the fifth stress day – SD5; the seventh stress day – SD7). Different letters indicate statistically significant differences among treatment means tested by ANOVA with Tukey's post hoc test (P < 0.05) within each cultivars and stress day, separately. Values are expressed as the mean ± standard deviation (n = 5).

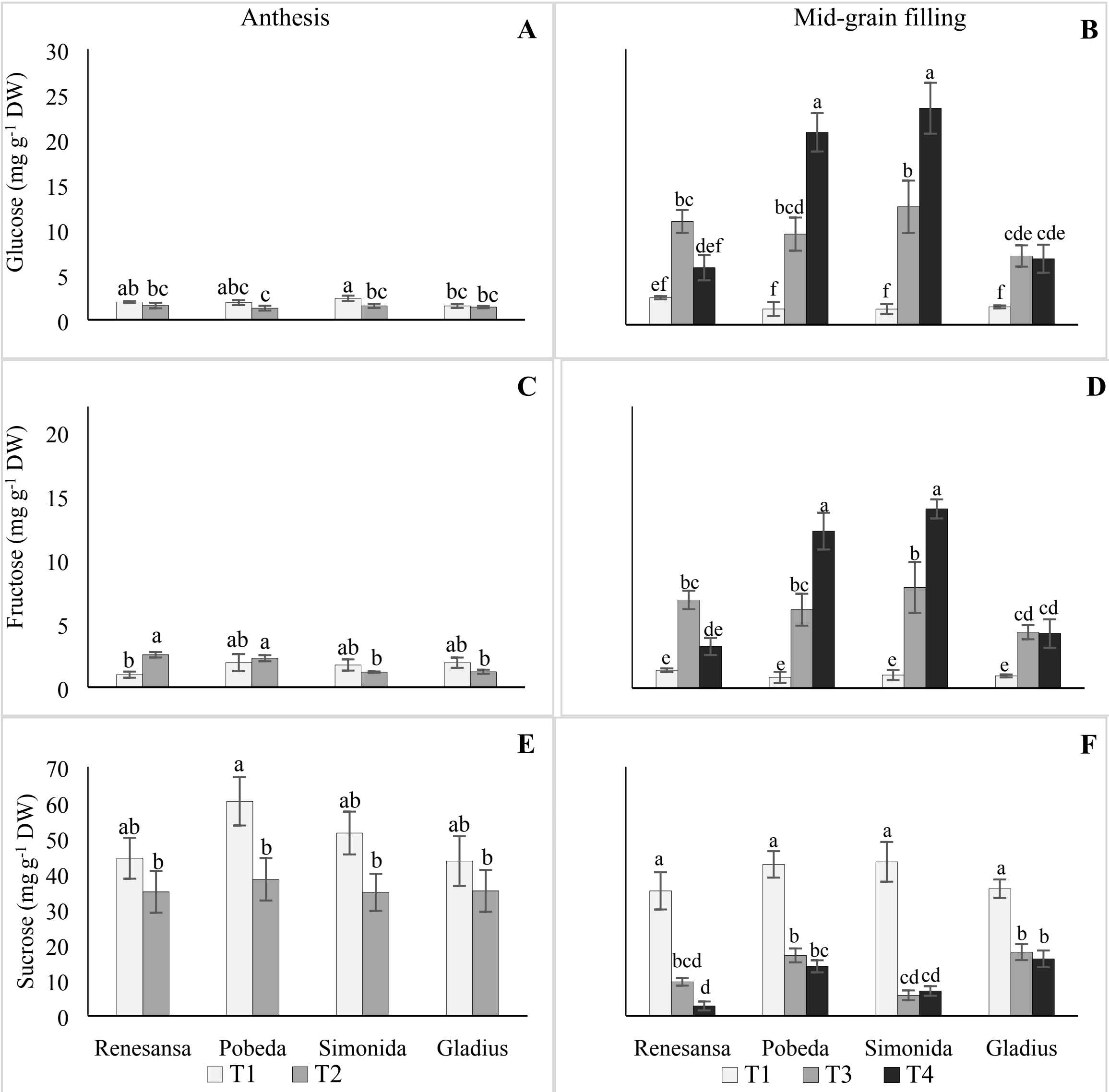


Figure 4. Carbohydrate analysis (glucose – A and B fructose – C and D; glucose – E and F) of the second stem flag leaf taken at the seventh stress day during anthesis (T1 – control anthesis; T2 – the heat stress at anthesis) and at the seventh stress day during mid-grain filling period (T1 – control mid-grain filling; T3 – heat stress mid-grain filling; T4 – combined stress at anthesis and mid-grain filling) in four winter wheat cultivars Renesansa, Pobeda, Simonida and Gladius. Different letters indicate statistically significant differences among treatment means tested by ANOVA with Tukey's post hoc test ($P < 0.05$). Values are expressed as the mean ± standard deviation ($n = 3$).

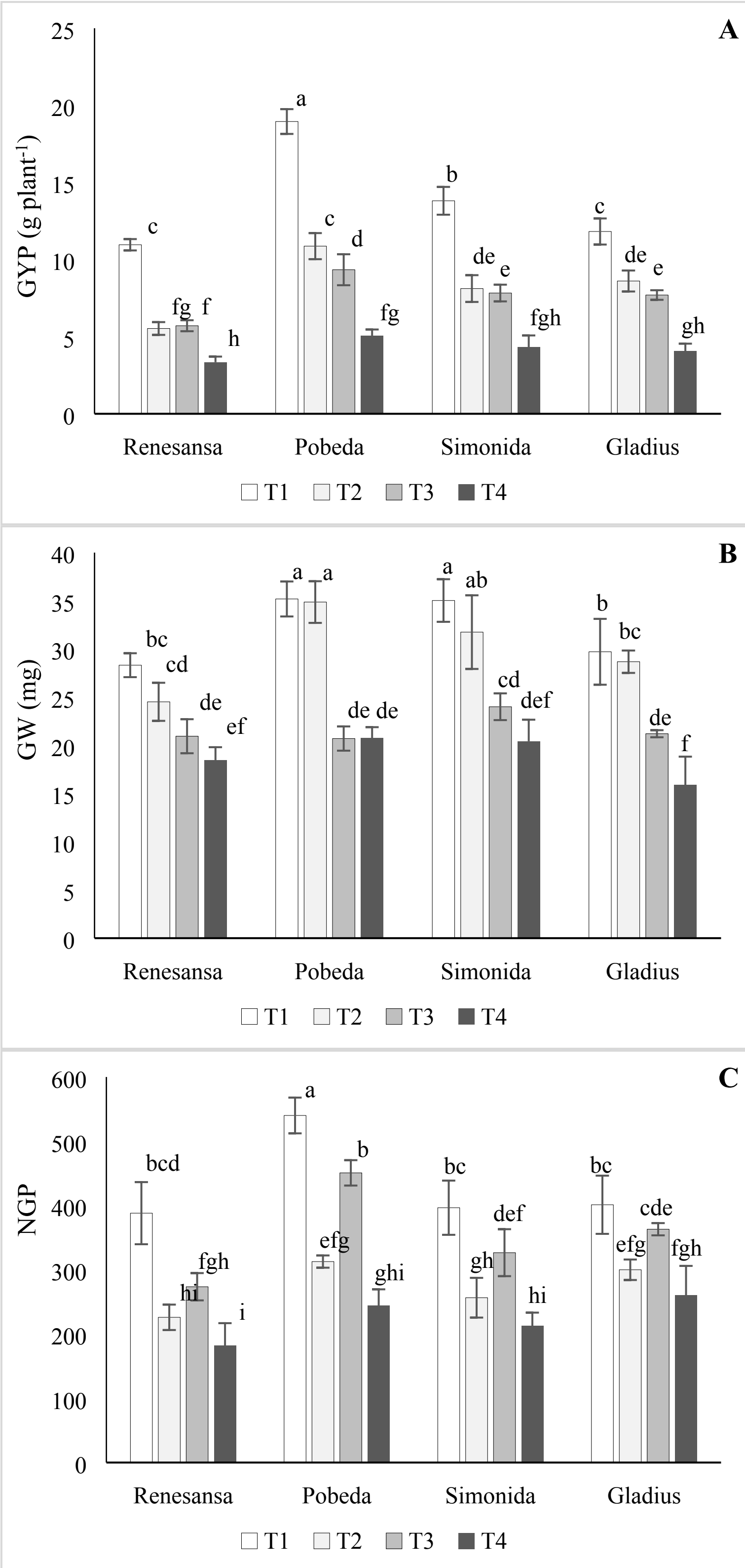


Fig. 5. Grain yield per plant (GYP; A), grain weight (GW; B) and number of grains per plant (NGP; C) in four winter wheat cultivars Renesansa, Pobeda, Simonida and Gladius grown under control conditions (T1), and seven days during the heat stress at anthesis (T2), heat stress at mid-grain filling (T3) and combined stress at anthesis and mid-grain filling (T4). Different letters indicate statistically significant differences among cultivar and treatment means tested by ANOVA with Tukey's post hoc test (P < 0.05). Values are expressed as the mean ± standard deviation (n = 5).