Periodic Determination of Physical and Physiological Responses to High Temperature Stress in cv. Narince Grapes

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Submitted for publication: July 2023
Accepted for publication: January 2024

Key words: *In vitro*, abiotic stress, MDA, viability, ion flow

Stress is a condition that affects or even inhibits growth, development and metabolism in plants. The type of stress, duration of application and severity also affect plants significantly. In this study, the weekly responses of Narince, a wine grape variety, to high temperature stress (HTS) in in vitro conditions were followed and stress was applied in two different periods, for 12 and 18 hours at 40°C. Some growth development (viability, fresh shoot weight, dry shoot weight, number of leaves, shoot length), physiological (ion flow and relative water content (RWC)) and biochemical (malondialdehyde (MDA)) analyses were done, with weekly follow-ups after the applications. In addition, the damage was graded visually. At the end of the application of both stresses, the percentage viability rate started to decrease. This rate decreased more slowly during HTS for 12 hours, while a faster decrease was observed in HTS for 18 hours. The number of healthy plants to be evaluated could not be reached after the fourth week. Growth and development parameters – fresh shoot weight (g), dry shoot weight (g) and shoot length (mm) – decreased after both stress applications, and these values were found to be statistically significant. After 12 hours of HTS application, the highest MDA value was determined at 0.274 nmol in the sixth week, while the lowest MDA value was determined in the first week, at 0.137 nmol. After 18 hours of HTS application, the highest MDA value was determined in the fourth week, at 0.263 nmol, while the lowest MDA value of 0.103 nmol was determined in the first week.

INTRODUCTION

Most species belonging to the genus *Vitis* are easily grown in many areas of the world where there are favourable climatic conditions. *Vitis vinifera* L., which is one of these species, is grown in many countries as table, dried and wine grapes due to its high adaptability to temperate climatic regions (Keller, 2015).

Successful viticulture depends on the complementary relationship between vine genetics and climatic factors (Keller 2015; Fraga *et al*., 2016). This has come about because individual species carrying the genes for tolerance to cold and/or hot climates have adapted to climates suitable for their genetic characteristics. However, the anomalies that occur in the climate from time to time affect the vines negatively (Köse & Güleryüz, 2009; Fraga *et al*., 2016).

Plants do not always have the optimum conditions necessary for their development, and since they do not possess a movement mechanism, such as moving away from the environment where the are located, the lack or excess of these conditions causes stress. Stress is examined in two ways: abiotic (drought, high/low temperature, salinity, etc.) and biotic (viruses, bacteria, etc.) stress (Levitt, 1980).

The temperature conditions of the environment where the plant is located affect plant growth and development significantly (Cramer, 2010). High temperature affects plant growth, metabolic activities and production, and is defined as the increase in temperature above the critical level for a certain period of time and is one of the most important abiotic stresses (Wahid, 2007; Hasaruzzaman *et al*., 2012).

The optimum daily temperature for photosynthesis, yield and fruit ripening in vine development is 30°C. It has been stated that heat stress can occur with an increase in temperature of 5°C in relation to optimum growth conditions in general (Keller, 2010). The rate of adaptation to high temperatures is high in vines. However, this adaptation is not unlimited, and it has been stated that the optimum leaf temperature for photosynthesis can increase up to 33°C (Schultz, 2000).

At high temperatures, cell damage and even death occur. The reason for this can be attributed to the destructive collapse of cellular organisation (Wahid *et al*., 2007). Many physical properties can be investigated as indicators of temperature damage. Some of the indicators of gas exchange in photosynthesis are net photosynthesis rate, stomatal conductivity, thermostability in membranes, ion flux and thiobarbituric acid-reactive substance content, and chlorophyll content (Xu *et al*., 2000; Rosyara *et al*., 2010).

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Acknowledgements: This project was supported by the Tokat Gaziosmanpaşa University BAP commission with the project number 2018/95

The response of the vine to high temperature stress is one of the most important issues in vine biology and is important for the sustainability of viticulture. In this study, it was aimed to observe the growth-development, physiological and biochemical changes of Narince, one of the grape varieties with a high wine value, under in vitro conditions with high temperature stress by monitoring these changes weekly.

MATERIALS AND METHODS

Material

The experiment was carried out by planting micro-cuttings obtained by maintaining the buds of the Narince grape variety. The explants were obtained from the Narince vineyard of the Agricultural Research and Application Farm of Tokat Gaziosmanpaşa University using the method of continuing the dormant cuttings. Cuttings were taken when dormant in February and maintained in the plant growth chamber.

Method

In vitro propagation

In vitro clonal propagation of the cultivar used in the study was carried out by the axillary bud culture method in accordance with Sivritepe (1995). The aim of the *in vitro* clonal propagation process is to propagate a sufficient number for the experiment in shoot information. The shoots, which developed five to six buds in the plant-growing room, were taken from the end and made into one-bud shoots (micro-cutting) and brought to the tissue culture laboratory for surface sterilisation in autoclaved jars containing pure water. The micro-cuttings brought to the laboratory were washed under running water for 20 minutes, with one or two drops of Tween 20 being added at intervals of 10 minutes. The micro-cuttings, which were kept in 70% ethyl alcohol for 15 seconds, were then moved to a sterile cabinet and rinsed with pure water. Here, the surface sterilisation process was continued, and the micro-cuttings were kept in 0.5% sodium hypochlorite solution twice for 10 minutes, then passed through distilled water three times and kept in the third distilled water, which concluded the process (Sivritepe, 1995). MS nutrient medium (Murashige and Skoog, 1962; MS; M-5519i Sigma Chemical Co.), which is preferred for plant tissue cultures, was used as the basic nutrient medium. To this nutrient medium was added 0.5 mg/l BAP (6-benzylaminopurin; B3408, Sigma), 3% sucrose and 0.7% Bacto agar. The pH of the nutrient medium was adjusted to 5.8 without adding agar (Sivritepe, 1995). Regarding the shoots, 15 ml of nutrient medium was put into 105 cc jars and autoclaved at 121ºC under 1.06 bar pressure for 20 minutes. One explant was planted in each jar. Stress treatments were started three weeks after planting.

Applications of high temperature stress

Heat stress was gradually increased to 40°C, which was the application temperature, for the explants in the plant growth cabinet, and the cycle was adjusted to 16 hours light and eight hours dark photoperiod at 25°C. The explants were exposed to high temperature stress at 40°C for 12 and 18 h.

After the stress treatments, the plants were brought back to the plant growth room of the tissue culture at 25°C, with a 16-hour light and eight-hour dark photoperiod. The parameters examined were taken at 7-day intervals for 6 weeks, and data collection started on the day the stress applications ended. Growth-development and physiological parameters were recorded, and the samples for biochemical analysis were stored at -20°C until the date of analysis.

Observations and analyses

Growth development parameters

The percentage viability – the number of explants that survived after the high-temperature application – was determined. Shoot fresh weights (g) were obtained by weighing using precision scales sensitive to ± 0.001 g. shoot dry weights (g) were subsequently determined using a precision scale with ± 0.001 g sensitivity after drying in an oven at 65°C for 72 h. The shoots length were measured using a digital calliper. The number of leaves/shoots (pieces) was the total number of leaves on each shoot (Dalsou & Short, 1987).

Physiological parameters

In terms of colour characteristics, the explants were removed from the nutrient medium after the applications and leaf colour was measured using a Minolta portable chromameter (Minolta, Model CR-400).

Explant water content was determined using the formula for relative water content (RWC) (%): [(FW-DW) / (TW-DW) × 100] (Yamasaki & Dillenburg, 1999). Using the aforementioned formula, fresh weights (FW) and turgor weights (TW) were determined after keeping them in distilled water for six hours, and dry weights (DW) were determined after 24 h of storage at 80ºC.

The ion flux (%) was determined using the method of Ozden *et al*. (2009) using leaf samples divided into equal parts. These samples of 0.3 g were placed in 25 mm × 150 mm glass tubes, to which 15 ml of distilled water was added. The prepared samples were shaken at 100 rpm for 24 hours in a shaker. After incubation, the electrical conductivity (EC1) of the solution was measured using an EC meter. Then the same samples were autoclaved at 115°C for 10 minutes. After the samples were kept at room temperature for 24 hours, the electrical conductivity (EC2) value of the solution was measured again. The ion flux in the leaves was calculated as EC1/EC2 × 100 and expressed as %.

Determination of temperature damage

The degree of damage to the explants (1 to 4) was determined according to Topcu Altıncı (2016). The plant shoots and leaves that were not damaged were graded as zero degrees; those with yellowing and drying up to 25% of the leaves and shoots were rated as one degree; those with yellowing and drying in 50% of the leaves and shoots of the plants were rated as two degrees; those with yellowing and drying in 75% of the leaves and shoots of the plants were rated as three degrees; and those with yellowing and drying in 100% of the leaves and shoots of the plants (dead plants) were rated as four degrees.

Biochemical analysis

Lipid peroxidation (MDA) was estimated by measuring the concentration of thiobarbituric acid reactive substances

(TBARS) using the thiobarbituric acid method described by Heath and Packer (1968). A total of 0.3 g of tissue was homogenised in 3 mL of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 x g for 10 min and 3 mL of 20 % TCA containing 0.5 % (w/v) 2-thiobarbituric acid (TBA) was added to 1 mL of supernatant. The mixture was heated at 95°C for 30 min and the reaction was stopped by quickly placing it in an ice bath. The cooled mixture was centrifuged at 10 000 x g for 10 min, and the absorbance of the supernatant was read at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the TBARS concentration was determined by its extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

The experiment was carried out according to the randomised plot design. In the high-temperature stress applications of the trial, three replications were found at each application dose and 40 explants were used in each replication. The data were analysed with ANOVA by using SAS Version 9.1 software (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was used to separate treatment means when ANOVA showed significant differences among the means. The level of significance was set at 5%.

RESULTS AND DISCUSSION

Statistical evaluation of the weekly data obtained after 12 and 18 hours of HTS applications at 40°C in the Narince cultivar grown in vitro are given in the tables below. After 12 hours of HTS, a six-week evaluation was made, and in the 18-hour HTS application, it was terminated after four weeks due to an insufficient number of live plants.

After the applications, viable explant counts were made in 10 of the explants removed from the plant growth chamber (Table 1). The percentage vitality (%) values, which were evaluated on a weekly basis after 12 and 18 hours of HTS applications, were found to be statistically significant. The highest number of viable explants was detected after 12 hours of HTS stress, with 90% in the first week, and the lowest in the sixth week, at 70%. The highest number of viable explants was detected after 18 hours of stress, with 80% in the first week, and the lowest was detected in the fourth week, at 40% (Table 1). A gradual decrease in vitality was encountered in the 12-hour HTS, while the 18-hour HTS experienced a higher and faster decline.

In terms of shoot length (mm), there was a statistically significant difference ($p < 0.05$) over weeks in the result of HTS applied for 12 hours (Table 1), while there was no statistical difference in the application of HTS for 18 hours (Table 1). After 12 hours of HTS, the longest shoot length was measured at 36.316 mm in the first week, while the shortest shoot length was measured at 22.016 mm in the sixth week. In terms of the number of leaves (number), there was no statistically significant difference on a weekly basis as a result of HTS applied for 12 and 18 hours ($p < 0.05$) (Table 1).

The explants, which were removed from the plant growth chamber every week, were removed from the medium in which they were found, and their shoot fresh weight (g) was weighed. According to these values, 12 and 18 hour HTS applications led to statistical differences ($p<0.05$) in shoot fresh weight (g) on a weekly basis. After 12 hours of HTS applications, the highest shoot fresh weight was measured with 0.990 mg in the second week, while the lowest fresh weight was measured at the sixth week with 0.354 mg. In the 18 hour HTS applications, the highest wet weight was measured with 0.506 grams in the wet shoot weight in the second week, and the lowest weight was determined with 0.351 mg in the fourth week (Table 1).

In terms of shoot dry weight (mg), there were statistical differences ($p < 0.05$) on a weekly basis as a result of HTS applied for 12 hours (Table 1), while there was no statistical difference in relation to 18 hours of HTS application. According to Table 1, the highest shoot dry weight after 12 hours of HTS was measured at 0.358 g in the second week, while the shoot dry weight was measured at 0.042 mg in the sixth week.

TABLE 1
Some growth development parameters after 12 and 18 hours of HTS applied to the Narince variety at 40°C.

	App.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
% Viability	**12 h**	A 100 a	A 100 a	A 90 b	A 90 b	80 c	70 d
	18 h	B 80 a	B 60 b	B 50 c	B 40 d	-	-
Shoot length (mm)	**12 h**	A 36.316a	A 34.790ab	A 34.943 ab	A 29.080 bc	28.290 c	22.016 d
	18 h	A 28.636 a	A 26.130 a	A 27.033 a	A 28.453 a	-	-
Number of leaves/ shoots (piece)	**12 h**	A 8.66 a	A 7.33 a	A 7.00 a	A 7.66 a	6.66 a	5.33 a
	18 h	B 5.00 a	A 5.66 a	A 7.00 a	A 5.66 a	-	-
Shoot fresh weight (g)	**12 h**	A 0.858 a	A 0.990 a	A 0.734 a	A 0.402 b	0.398 b	0.354 b
	18 h	B 0.501 a	B 0.506 a	B 0.434 a	B 0.351 b	-	-
Shoot dry weight (g)	**12 h**	A 0.358 a	A 0.387 a	A 0.126 b	A 0.060 c	0.052 c	0.042 c
	18 h	B 0.074 a	B 0.062 a	B 0.048 a	A 0.030 a	-	-

The different lowercase letters and capital letters indicate statistically significant differences between the treatments ($p < 0.05$)

 DOI: https://doi.org/10.21548/45-1-5906

In order for plants to survive under adverse environmental factors, the specific optimum environmental demands of that plant species must be met. Any increase or decrease in these optimum demands creates stress for the plant. In other words, stress is a condition that affects or inhibits metabolism, growth and development in plants (Levitt, 1980). Stress is generally one of the growth parameters of the plant; viability is measured based on productivity, growth (biomass accumulation) and primary extraction processes (CO_2 and mineral uptake) (Zeiger & Taiz, 2006). The decreases in the growth and development of plants because of stress are shown in Table 1. Viability (%) decreased in parallel with the duration of high temperature stress. The results show that the duration of the stress is as effective as its severity, and it also causes the plants' ability to regenerate to decrease. When damage from high temperatures exceeds the ability to regulate adversity, severe heat damage symptoms and even plant death may occur (Zha *et al*., 2018; Xu *et al*., 2020). Topcu Altıncı (2016) also reported decreases in fresh shoot and dry shoot weight, shoot length and leaf number in high temperature stress studies of six different wine grape varieties.

Table 2 shows the statistical evaluation of the physiological parameters of HTS applied for 12 and 18 hours at 40°C in the Narince cultivar grown in vitro. In terms of relative water content (%), there was no statistically significant difference when the two applications were evaluated on a weekly basis. However, as the weeks progressed, it was observed that this parameter decreased in both applications.

The RWC is an important factor in determining the effects of changing temperatures (Mazorra *et al*., 2002). The RWC is accepted as an important measurement method to determine the water potential of the plant in terms of reflecting metabolic activity in the tissues (Bertamini *et al*., 2006; Chylinski *et al*., 2007). Xiao *et al*. (2017) determined that the high temperature stress applied to the saplings of the Hongti grape variety at 38°C and 40°C and on different days affected the proportional water content significantly and, as the high temperature levels increased, the RWC in the leaves decreased significantly. They also reported that the primary reason may be that HTS caused a reduction in hydraulic conductance, leading to a decrease in water absorption.

When the L* value is 0, it means that the colour of the grain shell is black, that is, there is no reflection, while when the L* value is 100, it means that the colour is white, that is, the reflection is complete. In the "a*" value, negative values indicate green (-60: green) , while positive values indicate red (+60: red). In Table 2, there is no statistical difference based on the weekly evaluation of 12 and 18 hours of HTS application for the values of L and a, and based on 12 hours of HTS application for the b value. The value "b*" denotes the position between the colours yellow and blue, with negative values denoting blue, and positive values denoting yellow (-60: blue, +60: yellow). Only in the 18-hour HTS application of the b value did statistical differences ($p < 0.05$) emerge on the basis of the number of weeks.

In relation to the ion flow rate (%), there were statistical differences ($p < 0.05$) on a weekly basis when HTS was applied for 12 hours (Fig. 1), while there was no statistical difference in the application of HTS for 18 hours (Fig. 1). After 12 hours of HTS, the highest ion flow rate (61.38%) was determined in the sixth week, while the lowest rate was measured in the second week, at 24.42%. After 18 hours of HTS, the highest ion flow rate (50.33%) was measured in the fourth week, while the lowest rate was measured in the first week (32.11%).

In Fig. 2, the MDA values are given after 12 and 18 hours of HTS applied at 40°C. In terms of MDA (nmol), it is seen that there are statistical differences ($p < 0.05$) on a weekly basis as a result of HTS applied for 12 and 18 hours (Fig. 2). After 12 hours of HTS application, the highest MDA value was determined in the sixth week, at 0.274 nmol, while the lowest MDA value was determined in the first week, at 0.137 nmol. After 18 hours of HTS application, the highest MDA value was determined at 0.263 nmol in the fourth week, while the lowest MDA value was determined at 0.103 in the first week.

Cell damage and even death can occur at high temperatures. The reason for this can be attributed to the destructive collapse of cellular organisation (Wahid *et al*., 2007). Many physical properties can be investigated as indicators of temperature damage. Some of those are gas exchange indicators in photosynthesis, net photosynthesis rate, stomatal conductivity (Berry & Bjorkman, 1980;

TABLE 2
Some physiological parameters after 12 and 18 hours of HTS applied to the Narince variety at 40°C.

	App.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
*RWC (%)	**12 h**	A 92.41 a	A 89.60 a	A 86.76 a	A 74.48 a	73.63 a	64.50 a
	18 h	A 81.09 a	A 77.93 a	A 70.67 a	A 65.65 a	-	-
L *	**12 h**	A 37.72 a	A 34.03 a	A 34.03 a	A 39.40 a	35.90 a	38.96 a
	18 h	A 35.24 a	A 36.71 a	A 30.93 a	A 38.48 a	-	-
a*	**12 h**	A -10.52 a	A -11.18 a	A -11.83 a	A -11.81 a	-13.11 a	-12.57 a
	18 h	A -9.92 a	A -11.32 a	A -10.41 a	A -10.44 a	-	-
b*	**12 h**	A 18.06 a	A 14.68 a	A 15.82 a	A 17.93 a	15.12 a	18.13 a
	18 h	A 16.06 ab	A 17.80 a	A 12.55 b	A 16.49 ab	-	-

The different lowercase letters and capital letters indicate statically significant differences between the treatment ($p < 0.05$)

Stafne *et al*., 2000, 2001; Herzog & Chai-Arree, 2012), thermostability in membranes, ion flux, thiobarbituric acid-reactive substance (TBARS) content and chlorophyll content (Rosyara *et al*., 2010; Xu *et al*., 2000).

The cell membrane is thought to be the primary physical site damaged by heat stress (Basra *et al*., 1993). This damage arises on the leaf tissue, and high stress weakens the cell membrane. In this situation there is a tendency for ion flow out of the cell. For this reason, the measurement of ion flow is widely used to determine temperature damage (Wahid *et al.*, 2007).

MDA is a biochemical that tends to increase under many stress factors. Wang *et al*. (2004) and Topcu Altıncı (2016) have mentioned the increase in the amount of MDA under the conditions of high temperature stress to which they submitted grape varieties. MDA (lipid peroxidation) is an analysis method used in the biochemical evaluation of the stress factor in many stress studies. Lipid peroxidation can be viewed as the end product of essential cell membrane reactive damage to cellular mechanisms (Ali *et al*., 2005; Liu *et al*., 2006), and the MDA content is an important indicator of the extent of membrane lipid peroxidation. Most importantly, the accumulation of MDA can further damage the membrane and cells (Chen, 1989). These results show that high temperature stress causes significant increases in MDA and ion flow. In his study, Topcu Altıncı (2016) reported that the damage increased by showing the increase in MDA and ion flux values with the increase in heat stress. According to Xiao *et al*. (2017), high heat stress applied to grapevine saplings causes an increase in MDA and relative electrical conductivity (REC). These authors state that the reason for this increase is that high temperature disrupts the dynamic equilibrium between the production and removal of free radicals, which increases membrane lipid oxidation. It has also been reported that the increase in MDA content and its association with membrane protein causes membrane disruption and loss of function, increased plasma membrane permeability and, as a result, electrolyte leakage.

After 12 and 18 hours of HTS application at 40°C, 10 explants were used to visually assess the damage. Subsequently, the values obtained were expressed as percentages. Figs 3 and 4 show the proportional expression of the visual evaluation of the damage after the HTS application. In addition, the visual form of the rating is given in Fig. 5. No damage was observed on the plants in the first three weeks after 12-hour HTS application. As the weeks progressed, damage began to occur, and in the fifth and sixth weeks, degree 4 (plant death) was observed. After 18 hours of HTS application, degrees 0 and 1 were not recorded for 4 weeks. Degree 4 was detected in plants starting from the second week, reaching 40% damage in the fourth week.

CONCLUSIONS

This study aimed to examine the weekly course of high temperature stress in the Narince grape variety. The greenhouse effect caused by the CO_2 released into the atmosphere since the industrial revolution has been observed to have effects of high temperature, which pose

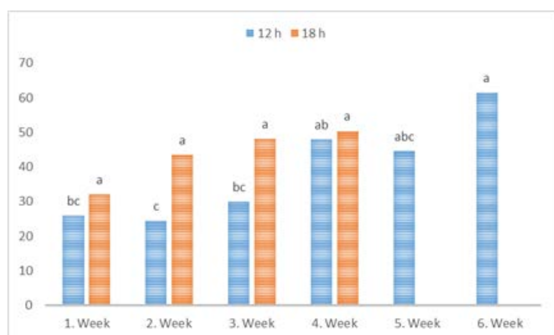

FIGURE 1
Ion flow (%)

FIGURE 2
MDA (nmol)

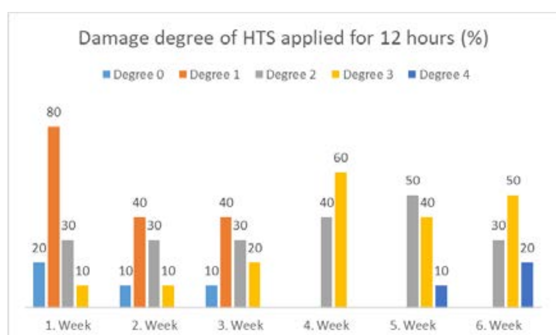

FIGURE 3
Visual evaluation of damage after 12 hours of HTS treatment applied to the Narince variety at 40°C (%).

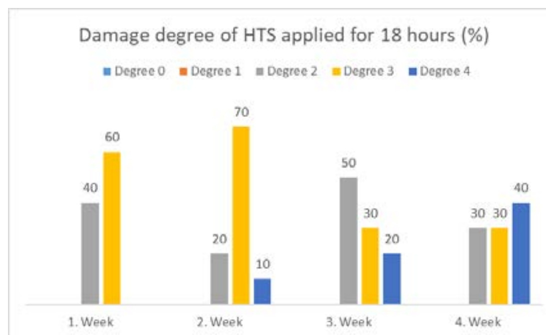

FIGURE 4
Visual evaluation of damage after 18 hours of HTS treatment applied to the Narince variety at 40°C (%).

 DOI: https://doi.org/10.21548/45-1-5906

FIGURE 5
Photographs of the visual evaluation of damage after 12 and 18 hours of HTS treatment applied to the Narince variety at 40°C.

a threat to grape varieties. In this study, the morphological, physiological and biochemical effects of high temperature stress on plants were investigated. It was determined that the stress caused decreases in viability (%), shoot length (mm), and in shoot fresh and dry weight (g). No statistical differences were observed in the number of leaves, relative water content or changes in colour. While a gradual increase in ion flux (%) was observed in the treatment with 12 hours of HTS, no statistical difference was observed in the 18-hour HTS treatment. MDA content increased with stress to a statistically significant level. The results obtained showed that high temperature stress is not only recorded in climate change scenarios. The intensity of physical, physiological and biochemical damage to plants must be determined and the responses of plants should be analyzed. It is known that the type of stress, along with its duration and amount, are important in stress applications. In the light of these results, we need to develop studies that can offer solutions to the problems that high temperatures, which will be felt more intensely in the coming years, will create in plant production.

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