South African Journal of Botany 147 (2022) 391–399

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Interactive effect of boric acid and temperature stress on phenological characteristics and antioxidant system in *Helianthus annuus* L.

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ARTICLE INFO

Article History:
Received 15 December 2021
Revised 17 January 2022
Accepted 27 January 2022
Available online 8 February 2022

Edited by M. Kulkarni

Keywords:
Abiotic stress
Antioxidant enzyme
Gene expression
Phenological parameters
Sunflower

ABSTRACT

Plants have to cope with more than one abiotic stress at the same time in nature. Heat stress and mineral toxicity are at the forefront of these abiotic stresses. The purpose of this study is to investigate the interactive effects of high and low-temperature stress and boric acid application on the sunflower and reveal the potential protection mechanisms against the combined effect of two types of stress. Two different boric acid concentrations (10 and 25 mM) were applied to sunflowers grown separately at low (15 °C), optimum (25 °C) and high (40 °C) temperature. Then, root length and stem height, root and stem fresh-dry weight, root and stem biomass, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) enzyme activities and changes in the gene expression levels of these enzymes were determined. It was determined that there were reductions in the phenological properties of the roots and stems of the samples grown at 40 °C based on the increasing boric acid concentration, while the samples grown at 15 °C were positively affected. The antioxidant enzyme activities and mRNA levels of the root and leaf samples at 40 °C were observed to increase. For the samples at 15 °C, the antioxidant enzyme activities and mRNA levels increased in the roots and decreased in the leaves. Considering phenological parameters, it was revealed that high boric acid application at low temperatures promoted plant growth, and considering the antioxidant enzyme activities and mRNA expression levels, it was highly effective in the plant's toleration of low temperatures.

1. Introduction

Sunflower is one of the most significant industrial plants in the world that is being produced in more than 70 countries with suitable climate conditions. Soil temperature must be at least 8–10 °C in order for sunflower seeds, which is a summer-growing plants, to germinate.The optimum temperatures for development are between 21–24 °C. Higher or lower temperatures than suitable temperatures affect germination, growth, and flowering yield (Gay et al., 1991).

Global warming, which is at the top of the ecological problems of our time, leads to a worldwide temperature increase and drought based on this increase. Agricultural losses and changes in plant yield and product quality in the world are attributed to changes in temperature in addition to other stressors, especially drought (Kotak et al., 2007; Wahid et al., 2007; Hasanuzzaman et al., 2013; Li et al., 2014). In this context, one of the most significant abiotic factors is temperature. Temperature stress disrupts the cellular homeostasis, leads to significant retardation in growth development, and may even lead to the death of the plant. Some metabolic events that are caused by high temperatures and directly affect the plant may be listed as inhibition of protein synthesis by protein denaturation, increased fluidity of membrane lipids, loss of membrane integrity, inactivation of enzyme in the chloroplast and mitochondria, and imbalance between photosynthesis and respiration (Wahid, 2007). In turn, these negative effects lead to hunger, growth inhibition, reduced ion flow, and increased toxic compounds and reactive oxygen species (ROS) in the plant (Sairam et al., 2000; Wahid et al., 2007).

As much as high temperatures, low temperatures also create stress in plants and lead to negative effects on the development of plants (Hussain et al., 2018). Low-temperature stress leads to serious yield losses by delaying plant development (Ruelland et al., 2009; Hussain et al., 2018). Low temperatures stress disrupts the osmotic balance of the plant and also leads to dehydration stress (Farooq et al., 2009; Wang et al., 2016). As in most stress cases, ROS are formed also in low temperature stress, and excess ROS production leads to oxidative damage (Farooq et al., 2009; Ruelland et al., 2009). Excess ROS accumulation leads to protein oxidation, peroxidation of membrane lipids, DNA and RNA damage, and even cell death (Apel and Hirt, 2004).

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https://doi.org/10.1016/j.sajb.2022.01.039
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Insufficiency or excess intake of nutrients affects plant growth and leads to losses in yield and product quality. One of the most important micronutrients for plants is boron. Boric acid is a soluble compound that is easily leached by precipitation; therefore, boron deficiency is usually seen in humid regions. In contrast to boron deficiency, soil boron toxicity is less observed and occurs in arid and semi-arid areas. Although the role of boron in physiological pathways and molecular responses has not been completely understood yet, it is known that boron plays a role in significant physiological and biochemical events in plants such as membrane integrity, enzyme reactions, transport of photosynthesis products to other parts of the plant, and the carbohydrate and phenol metabolism (Cervilla et al., 2009; Barut et al., 2018). The primary phenotypic effect of boron toxicity is generally a reduction in the plant dry weight and inhibition of root growth (Turan et al., 2009). Boron toxicity usually leads to oxidative stress causing accumulation of ROS like hydroxyl radicals (OH^-), superoxide radicals (O_2^-), and hydrogen peroxide (H_2O_2), and eventually, cell death. Since boron is a microelement, there are studies in the literature showing that when taken in sufficient quantities by the plant, it helps the plant to tolerate other abiotic stresses (Blamey et al., 1997; Waraich et al., 2011, 2012).

Abiotic stresses are rarely encountered alone in nature. As plants are affected by several stress factors at the same time throughout their lifespan, it is highly important to investigate the combined effects of stress factors. Based on this fact, this study aims to determine the interactive effect of i) high temperature and boron application, ii) low temperature and boron application on phenological parameters and antioxidative enzyme activity and gene expression in the sunflower plant. Interactive effects of different temperatures and different boric acid concentrations were investigated in this study.

2. Materials and methods

2.1. Growing of plant samples

In this study, seeds of Sy-Suzuka, which is the most frequently grown sunflower variety in Turkey, were used as the experimental material. The seeds were sterilized by using 10% NaClO. 6 sunflower seeds were sown into each of the 2.7-liter (L) pots, and the experiments were carried out with 3 replications for each treatment (control, 10 mM, and 25 mM boric acid) (Mahboobi et al., 2002; Karabal et al., 2003; Ye, 2004; Beyaz et al., 2018). The seeds were irrigated with distilled water for 5 days until their cotyledons emerged, and after the cotyledons emerged, the control group was irrigated with 150 ml of distilled water every other day, and the experiment groups were irrigated with 150 ml of 2 different boric acid solutions as 10 mM and 25 mM every other day. These experimental conditions were applied on plant samples grown at low (15/10 °C), optimum (25/20 °C), and high (40/35 °C) in a climate room adjusted to a photoperiod of 16/8 h and 60% humidity. The seedlings that were grown were collected after the 30th day after sowing. The root lengths and stem heights (cm) of plant samples were measured with a ruler and root-stem fresh-dry weights (g) were measured with precision balances. Root-stem biomass (g $m^{2\ -1}$) was calculated (Sulus and Leblebici, 2020). The experiment was performed as three dependent and three independent replicates.

2.2. Determination of lipid peroxidation

The lipid peroxidation (MDA) was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid reaction. In the study, the MDA contents of leaf samples were determined according to following the procedure as adopted by Kabay and Şensoy (2017) with minor adjustments.

2.3. Determination of Antioxidant Enzyme Activities

SOD determination kit was used to determine SOD activity (19,160 SOD Determination Kit, Sigma-Aldrich). CAT activity in the root and leaf samples was determined according to the method reported by Tepe and Aydemir (2011), while APX activity was determined by using the method of Cevilla et al. (2007) with slight modifications.

2.4. Determination of gene expressions of antioxidant enzymes

A Macherey-Nagel total DNA, RNA, and protein isolation kit (Macherey-Nagel, MN) was used for total RNA isolation from the root and leaf samples of the plants. The isolated RNA samples were converted into cDNA by using a cDNA synthesis kit (NucleoGene RNA to cDNA Mix (5X), Nucleogene). For quantitative real-time polymerase chain reaction (qRT-PCR) experiments, primers specific to each gene were designed, and the list of the primers is given in Table 1. The obtained cDNAs were used as templates for quantitative real-time PCR. Quantitative real-time PCR analysis was carried out by using a Biorad SsoFast EvaGreen Supermix kit manufacturer's instructions in a PCR device (Agilent). The PCR reaction was carried out with 3 replications with the primers of the *SOD-Mn*, *SOD-Fe*, *SOD-Cu/Zn*, *CAT*, and *APX* enzyme genes, and Actin gene was used as a reference gene.

2.5. Statistical analysis

Each study with control and experimental groups exposed to different temperatures and different boric acid concentrations was performed with at least 3 independent and 3 dependent replications. For statistical analysis, the p-value was calculated by using a two-way analysis of variance (Two-Way ANOVA) in the Graphpad program.

3. Results

3.1. Changes in phenological parameters

The thirth day after sowing, root and leaf samples were collected from the plants, root lengths, stem heights (cm), and fresh-dry weights of the root and stem (g), root-stem biomass (g $m^{2\ -1}$) were determined.

It has been determined that both high and low temperatures negatively affect the root length and stem height of the plant compared to the optimum temperature. Boric acid stress and heat stress increased the negative effect of high temperature on root and stem length, while it reduced the negative effect of low temperature. Interestingly, a positive effect of boric acid application at low temperature on the stem length of the plant was determined (Table 2).

When root fresh weight was examined, it was determined that both high-temperature stress and low-temperature stress

Table 1
Sequences of the primers that were used.

Primer Name	Sequence (5′ – 3′)	Tm
SOD-Mn (F)	5′-CTGGAAAGAATCTTGCCCCTACTCGT -3′	65
SOD-Mn (R)	5′- CAACCAATTTTTCCATAGAACCAAAAT G -3′	59
SOD-Fe (F)	5′- TAATGGCGTCTGTCACCACA -3′	57
SOD-Fe (R)	5′- TGTGCAGCATTGTTGAAGGC -3′	57
SOD-Cu/Zn (F)	5′- ACACAATCTTTGCAGGGGTGTTCTGAGA -3′	65
SOD-Cu/Zn (R)	5′- TGCAGTACCATCT TCGCCTACTGTGACA -3′	67
CAT (F)	5′- CTTCCCGCTTGAATGTGAAG -3′	64
CAT (R)	5′- CCGATTACATAAACCCATCATC -3′	60
APX (F)	5′- TGGCGATGCCTATTGTAGAC -3′	57
APX (R)	5′- TCCTCGCAAAAATCGATAGC -3′	55
Actin (F)	5′- AGGGCGGTCTTTCCAAGTAT -3′	64
Actin (R)	5′- ACATACATGGCGGGAACATT -3′	63

Table 2
Phenological data of root samples.

		Root Length (cm)	Root Fresh Weight (g)	Root Dry Weight (g)	Root Biomass (g m$^{2\ -1}$)
Low temperature (15 °C)	Control	16.15±0.42***	0.95±0.004***	0.05±0.04	0.87±0.011***
	10 mM	14.50±0.14**	0.73±0.1***	0.04±0.002	1.24±0.010***
	25 mM	14.33±0.04***	0.46±0.1*	0.03±0.001	1.24±0.172
Optimum temperature (25 °C)	Control	21.25±0.35	1.21±0.02	0.08±0.01	2.43±0.057
	10 mM	16.25±0.71	0.61±0.01	0.03±0.002	1.97±0.134
	25 mM	10.84±0.23	0.49±0.005	0.03±0.002	1.09±0.066
High temperature (40 °C)	Control	15.83±0.18***	0.73±0.04***	0.05±0.004	1.53±0.012**
	10 mM	10.63±0.18***	0.36±0.01***	0.03±0.001	0.96±0.018***
	25 mM	7.98±0.11***	0.21±0.001***	0.01±0.001*	0.37±0.006**

* 15 °C and 40 °C group compared with 25 °C experimental groups (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Table 3
Phenological data of stem samples.

		Stem Length (cm)	Stem Fresh Weight (g)	Stem Dry Weight (g)	Stem Biomass(g m$^{2\ -1}$)
Low temperature (15 °C)	Control	16.88±0.11***	1.92±0.07***	0.13±0.01	4.31±0.236***
	10 mM	18.43±0.32***	2.38±0.02***	0.21±0.002*	7.65±0.054***
	25 mM	23.30±0.28***	3.11±0.09***	0.29±0.002**	8.64±0.145***
Optimum temperature (25 °C)	Control	26.53±0.32	1,38±0.001	0.19±0.003	5.99±0.034
	10 mM	22.38±0.18	0.51±0.001	0.14±0.0	3.52±0.0
	25 mM	20.58±0.11	0.29±0.01	0.09±0.01	2.94±0.007
High temperature (40 °C)	Control	22.68±0.18***	1.60±0.03**	0.13±0.002	6.22±0.077
	10 mM	21.26±0.13*	0.29±0.01***	0.08±0.006*	4.34±0.051***
	25 mM	16.59±0.41***	0.11±0.004**	0.07±0.003	2.53±0.034**

15 °C and 40 °C groups compared with 25 °C experimental groups ($p<0.05$; ** $p<0.01$; *** $p<0.001$).

significantly reduced root fresh weight. Boric acid and heat stress significantly increased the effect of both high and low-temperature stress on the root fresh weight of the plant (Table 3).

It was determined that stem fresh weight decreased at high temperature and increased at low temperature, consistent with stem length. Also, consistent with the results in stem length, it was determined that boric acid application increased the negative effect of high temperature. It was determined that the negative effect of low temperature decreased and positively affected stem growth (Table 2).

In addition, it was found that high temperature and the boric acid application did not cause a significant change in root and stem dry weight. However, it was determined that low temperature and boric acid application significantly increased the stem dry weight and did not cause a significant change in root dry weight (Tables 2 and 3).

When root and stem biomass were examined, it was determined that high temperature significantly reduced root and stem biomass in connection with other findings, while boric acid application significantly increased the negative effect caused by high temperature. It was determined that the boric acid application in low temperature significantly increased the root and stem biomass (Tables 2 and 3). These findings demonstrate that boric acid application at low temperature has a very important positive effect on the phenological parameters of the plant stem.

3.2. Changes in lipid peroxidation

Lipid peroxidation is one of the most basic indicators of oxidative stress. To determine lipid peroxidation, MDA contents were determined from the root and leaf of plant samples. Interestingly, root-MDA content decreased at all concentrations at high temperature and at control and 10 mM concentration at low temperature, while it increased significantly at low temperature 25 mM. Leaf-MDA content decreased in all concentrations at low-temperature stress, and only in control and 10 mM boric acid application at high-temperature stress but increased significantly at high temperature 25 mM (Fig. 1a and 1b).

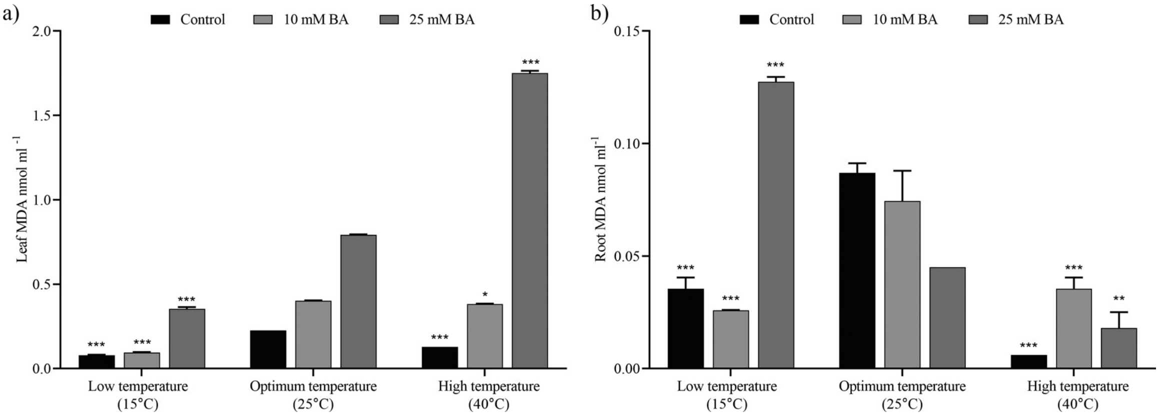

Fig. 1. MDA content of samples a) leaf, b) root (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

3.3. Changes in antioxidant enzyme activities

The antioxidant enzyme activity in the root was investigated. It was determined that high and low-temperature stress significantly increased the root-SOD activity and decreased the root-APX activity at all concentrations. It was determined that the application of high concentrations of boric acid in addition to the high and low-temperature stress applied separately further increased the root-SOD activity and decreased the root-APX activity (Fig. 2a, 2c). It was found that root-CAT activity increased only at high-temperature stress at all concentrations. In addition to high-temperature stress, the application of a high concentration of boric acid significantly increased the root-CAT activity compared to those applied only to heat stress (Fig. 2b).

It was determined that the leaf-SOD activity increased compared to the optimum temperature at both high and low temperature at the control and both concentrations. Different concentrations of boric acid applied with high-temperature stress increased the leaf-SOD activity more than only high-temperature stress. Also, it was determined that low-temperature stress and boric acid stress increased leaf-SOD activity more than only low-temperature stress (Fig. 3a). Leaf-CAT activity decreased at control and 10 mM boric acid concentration relative to optimum temperature at both low and high-temperature stress. Leaf-APX activity decreased at both concentrations at low and high temperatures. Boric acid stress was also applied to plants with individual high or low-temperature stress. It was determined that the synergistic effect of the two stresses together was greater than when they were applied alone and further reduced leaf-CAT and leaf-APX activities (Fig. 3b and 3c).

3.4. Changes in gene expressions of antioxidant enzymes

Root-*SOD-Mn* and root-*APX* gene expression increased significantly at 25 mM boric acid concentration both in high temperature-boric acid application and low temperature-boric acid application compared to optimum temperature (Figs. 4 and 6). Root-*CAT* gene expression in high temperature decreased significantly in 10 mM boric acid application compared to the optimum temperature, while it increased significantly in 25 mM boric acid application. In low-temperature stress, on the contrary, root-*CAT* gene expression increased significantly at 10 mM boric acid concentration compared to the optimum temperature, while it decreased significantly in 25 mM boric acid application (Fig. 5).

It was determined that leaf-*SOD-Mn* and leaf-*SOD-Cu/Zn* gene expression decreased significantly at both boric acid concentrations in low temperature-boric acid application compared to the optimum temperature, while leaf-*SOD-Fe* expression increased significantly at both concentrations in low temperature-boric acid application (Fig. 7a–7c). In the high temperature-boric acid application, leaf-*SOD-Mn* gene expression increased significantly at 25 mM boric acid concentration compared to the optimum temperature, while leaf-*SOD-Fe* expression decreased at 10 mM boric acid concentration and leaf-*SOD-Cu/Zn* expression decreased at both boric acid concentrations (Figs. 7a–7c). Leaf-*CAT* and leaf-*APX* gene expression were significantly decreased at both boric acid concentrations in both low temperature-boric acid application and high temperature-boric acid application compared to optimum temperature (Figs. 8 and 9).

4. Discussion

In plants exposed to temperatures high enough to damage tissues, observable changes take place in phenological properties. The harmful effects of high-temperature stress on the root system of the plant are typically characterized by reductions in the root biomass, the number of roots, and metabolic activities (Huang et al., 2012). Additionally, high-temperature stress leads to reduced photosynthesis, and therefore, limited plant growth (Wise et al., 2004). Boron toxicity, which was the other abiotic stress in our study, inhibits root growth by causing a cytotoxic effect on root tip cells during mitotic cell division. This situation is observed in the form of a reduction in the plant's dry weight and biomass (Turan et al., 2009). Considering the effects of two different boric acid concentrations on the samples in our study, it was observed that the root length and stem height significantly decreased by the increasing boric acid concentration in comparison to the control in the samples grown at optimum temperature. Studies have reported that the application of toxic amounts of boric acid reduces the root stem fresh-dry weight and biomass of the

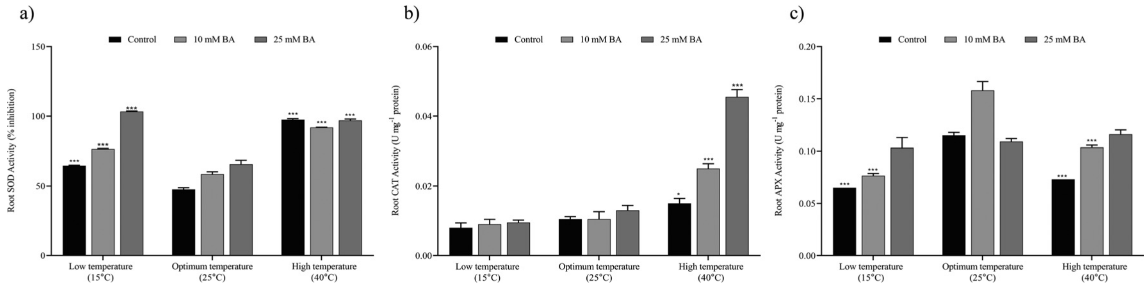

Fig. 2. Antioxidant enzyme activities of root samples a) SOD, b) CAT, c) APX (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

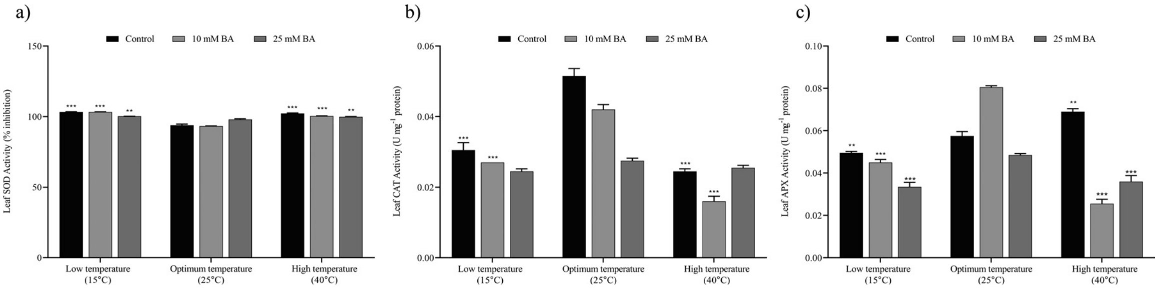

Fig. 3. Antioxidant enzyme activities of leaf samples a) SOD, b) CAT, c) APX (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

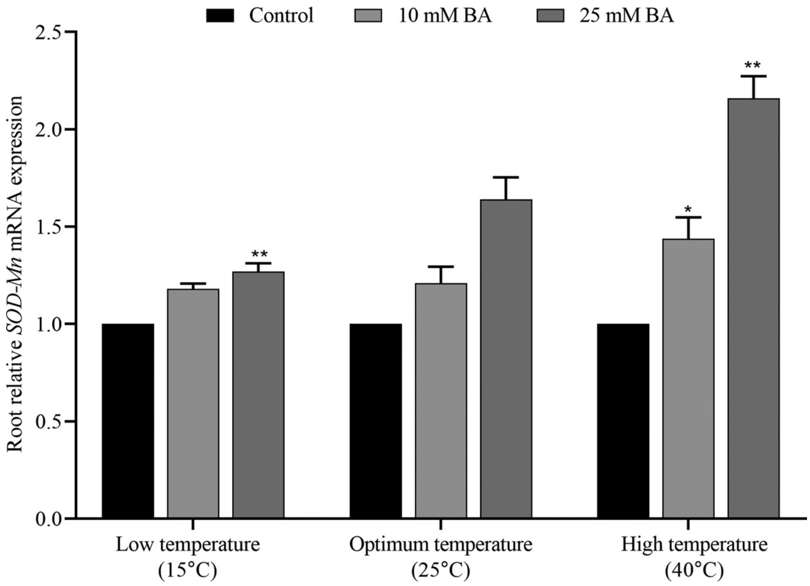

Fig. 4. *SOD-Mn* mRNA levels of root samples (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * p<0.05; ** p<0.01; *** p<0.001).

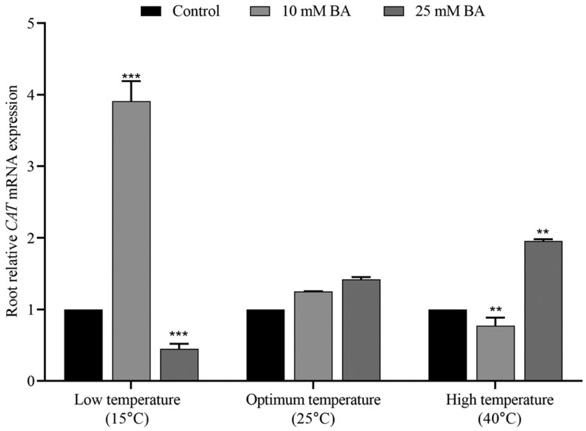

Fig. 5. *CAT* mRNA levels of root samples (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * p<0.05; ** p<0.01; *** p<0.001).

plant which is confirmed in this study (Blamey et al., 1997; Torun et al., 2006; Ardic et al., 2009; Irfan et al., 2019).

For revealing the combined effects of both high temperature and boric acid application, which made our study unique, our experiment groups grown at high temperature (40 °C) and those grown at optimum temperature (25 °C) were compared. It was determined that there was a gradual reduction in all phenological parameters of the plant samples grown at high temperature based on the increasing boric acid concentration (Tables 2 and 3). While there is no study in the literature in which boric acid and temperature stress are applied together in sunflower, there are very few studies in which other plants are used (Sarwar et al., 2019; Calderón-Páez et al., 2021). Nevertheless, other studies in the literature that were conducted only on temperature stress supported the results of our study on high-temperature stress. In parallel with our study, high-temperature stress led to a reduction in biomass amount and seed weight. It was revealed that, because of the combined effect of the high-temperature stress, the roots could not achieve the optimum development, and concerning this, they were not able to collect sufficient water and water-soluble nutrients from the soil. The reduction in the root and stem biomass may be explained by the lack of sufficient accumulation of the needed water and organic matter in the plant and a related reduction in the dry weight (Rahman et al., 2009; Bavita et al., 2012; Meena et al., 2016).

A high concentration of boric acid which leads to oxidative stress increases the reactive oxygen species in the plant, and based on this, it increases lipid peroxidation, as in the case of other abiotic stress factors. Under the optimum temperature conditions, plants establish a balance between the production and elimination of reactive oxygen species (Li et al., 2014). In this study, it was observed that the leaf-MDA content of the samples grown at high temperature increased gradually depending on the increasing boric acid concentration, while the root-MDA content decreased (Fig. 1a and 1b) as reported studies (Yin et al., 2008; Yong et al., 2008; Zhang et al., 2008; Bavita et al., 2012; Hussain et al., 2016).

Abiotic stresses such as drought, salinity, and cold cause an increase in ROS formation in plants due to disruption of cellular homeostasis (Mittler, 2002; Sharma and Dubey, 2005; Han et al., 2009; Mishra et al., 2013). It is known that high-temperature stress and boric acid toxicity separately also affects the activity of the antioxidant enzymes of SOD, CAT, and APX (Bavita et al., 2012; Li et al., 2014; Genisel et al., 2017). Studies in the literature declare that stem and root SOD, CAT, and APX activities increased by high-temperature stress (Bavita et al., 2012; Rani et al., 2013; Zeng et al., 2021) and boric acid stress separately (Esim et al., 2013; Genisel et al., 2017), on the other hand, some studies declare that SOD, CAT, and APX activities decreased by high-temperature stress (Liu and Huang, 2000; Yin et al., 2008; Li et al., 2014; Harsh et al., 2016; Afzal et al., 2020) and boric acid stress separately (Ardic et al., 2009; Tepe and Aydemir, 2011).

There are studies on different plants revealing the combined effects of different abiotic stress sources. When the combined effect of high temperature and boric acid stress was compared, it was observed that the root-SOD, leaf-SOD, and root-CAT activity increased, root-APX, leaf-CAT, and leaf-APX activity decreased (Figs 2a−2c and 3a−3c). It has been reported in studies that the combined effect of the two stresses increases the enzyme activity compared to the situation in which the stress is applied alone consistent with our results (Hussain et al., 2018; Demir, 2019; Raja et al., 2020).

Abiotic stresses cause excessive production of toxic reactive oxygen species (ROS), and the produced ROS cause serious damage to the organism at the cellular level. Therefore, plants have very efficient ROS scavenging mechanisms using enzymatic and non-enzymatic components. SOD, CAT, and APX are enzymatic components, and

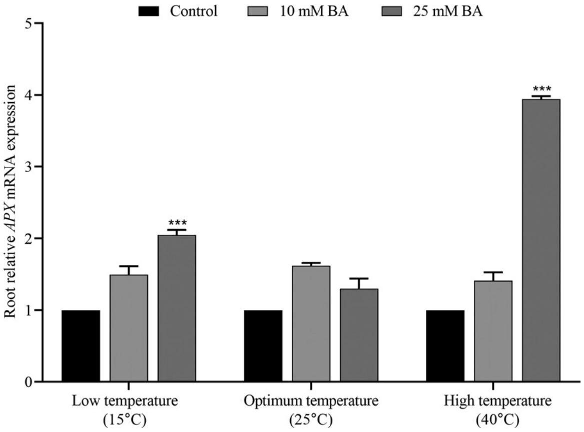

Fig. 6. *APX* mRNA levels of root samples (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * p<0.05; ** p<0.01; *** p<0.001).

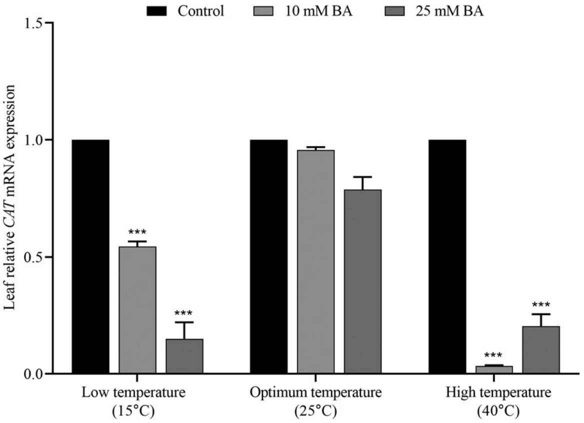

Fig. 8. *CAT* mRNA levels of leaf samples (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * p<0.05; ** p<0.01; *** p<0.001).

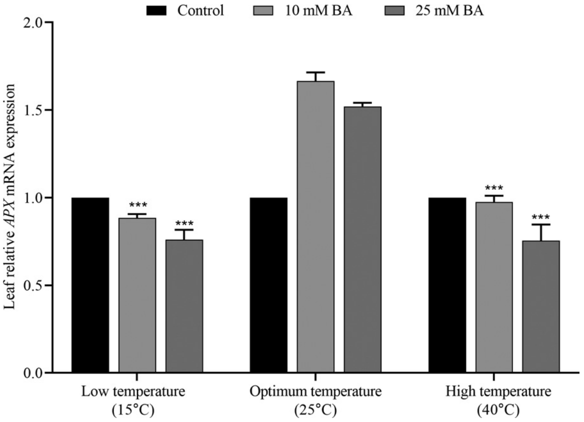

Fig. 9. *APX* mRNA levels of leaf samples (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * p<0.05; ** p<0.01; *** p<0.001).

gene expression levels are rearranged under abiotic stress (Filiz et al., 2019). Based on their metal cofactors, protein folding, and subcellular distribution, SODs are mainly categorized as SOD-Cu/Zn, SOD-Fe, and SOD-Mn. SOD-Cu/Zn can be found in the cytoplasm, chloroplasts, and intercellular space of prokaryotic and eukaryotic organisms. While SOD-Fe's are found in plant chloroplasts and cytoplasm, SOD-Mn's have been observed in eukaryotic mitochondria and protect mitochondria by scavenging ROS (Fan et al., 2014; Feng et al., 2016). The CAT enzyme represents one of the primary enzymatic defenses against oxidative stress and is mostly found in the cytosol and peroxisomes (Azpilicueta et al., 2008; Ara et al., 2013). It is important in all oilseeds and its activity has been shown to be closely related to the germination rate in sunflower (Azpilicueta et al., 2008). Similar to SOD and CAT, the APX enzyme is a component that helps the plant defend against oxidative stress and is found in the cytosol and mitochondria. In studies conducted with canola, wheat, barley, zucchini, tobacco, rice, and valerian high-temperature stress was applied on plants, and it was reported that the gene expression levels of antioxidant enzymes, especially *SOD, CAT,* and *APX*, increased (Sato et al., 2001; Ara et al., 2013; Zhao et al., 2014; Harb et al, 2015; Hosseini et al., 2015; Dudziak et al., 2019; Dai et al., 2020). Consistent with the literature, our results show that application of high-temperature stress alone increases root-*SOD-Mn*, root-*APX,* leaf-*SOD-Mn* gene expression and decreases leaf- *SOD-Fe*, leaf-*SOD-Cu/Zn*, leaf-*CAT,* and leaf-*APX* compared to optimum temperature (Figs. 3−6a−c and 7−9). In the literature, it has been reported that the synergistic effect of more than one stress on oxidative genes is greater than when applied alone (Raja et al., 2020). In our results, the application of boric acid to the plant with high temperatures further increases the negative effect of high temperature.

Plants that are exposed to cold stress reduce their cell production by extending their cell cycle. Reduced cell production causes a slower growth and a smaller leaf size in the plant (Rymen et al., 2007). Cold stress also limits the development of the plant. In plants like sunflower, temperatures in the range of 10−17 °C induce cold stress on the roots (Huang et al., 2005). In parallel with our results, it has been reported in the literature that root length and stem height, root and stem fresh weight, root development, and biomass decrease in maize, rice, and cucumber plants that are subjected to low-temperature stress (Farooq et al., 2009; Yan et al., 2012; Hussain et al., 2016, 2018).

The most striking results of our study were obtained in the combined effect of low temperature and high boric acid application. Interestingly, in the samples on which both low temperature and boric acid stresses were applied, the phenological parameters showed an increase in comparison to the optimum temperature along with the increasing boric acid concentration. In fact, considering the samples on which boric acid was applied, it was determined that the best root length and stem height, root fresh weight, stem

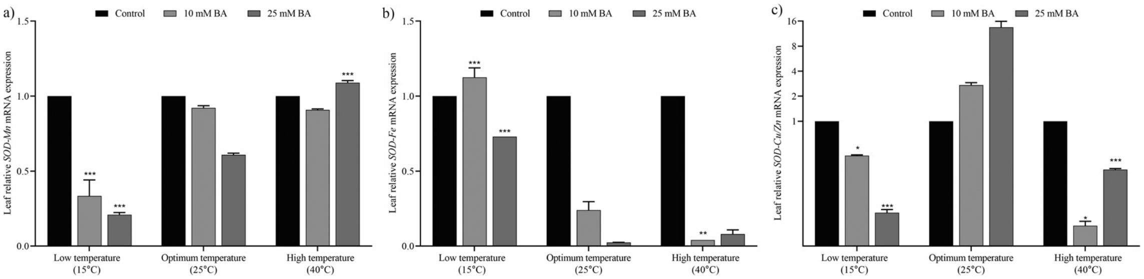

Fig. 7. *SOD* mRNA levels of leaf samples a) *SOD-Mn*, b) *SOD-Fe*, c) *SOD-Cu/Zn* (*; 15 °C and 40 °C groups compared with 25 °C experimental groups. * p<0.05; ** p<0.01; *** p<0.001).

fresh and dry weight, and stem biomass were found at low temperature. Therefore, the combination of low temperature and excess boron caused the samples to develop better in comparison to the optimum temperature.

In literature, one study determined that stem dry weight and the stem/root ratio increased in sunflower plants that were given boron supplementation at a low temperature (Ye, 2004). It was also stated that boron deficiency increases the damage rate in the plant under cold stress (Liu et al., 2016). This situation revealed that, as in our study, boron plays a role in protecting the plant against cold stress and increasing yield. In addition, in a study in the literature, a concentration of 6.5 μm boric acid was used, and it is seen that this concentration is quite low compared to our study (Ye, 2004). In sunflower plants exposed to cold stress, the hydraulic conductivity value of the roots decreases, and due to the disruption of stromal control, transpiration increases (Allen and Ort, 2001). It is stated that, due to cold stress, boron intake from the roots decreases, and this leads the plant to experience boron deficiency. It is known that roots cannot get enough boron in cold. In this study, the amount of boron in the plant was measured and it was reported that boron was taken from the roots at low temperatures, and accordingly, the amount of boron in the stem increased (Ye, 2004). The high boron level may explain boron intake from the roots and its transfer to the stem with increased diffusion, but the relationship between low temperatures and boron has not been explained on the molecular level yet. In our study, it was observed that applying a low temperature and a high concentration of boric acid led to a significant increase in the phenological parameters of especially the stem. This shows that boron application plays a role in the plant's toleration of low-temperature stress. The constant exposure of the roots to the water and boric acid accumulating in the soil may explain the result that the parameters related to root development were negatively affected.

There are also studies reporting that when one or more of the macro and microelements are given externally in addition to abiotic stress, it increases the plant's ability to cope with abiotic stress (Tavallali et al., 2018; Sarwar et al., 2019).

While MDA level was expected to increase with increasing oxidative stress, Leaf-MDA level decreased in with boric acid application in plants with low-temperature stress compared to optimum temperature. This result confirms the conclusion in the literature that the boron application can tolerate some abiotic stresses of the plant (Li et al., 2014; Jamshidi-Goharrizi et al., 2021) (Fig. 1a). In our study, the most striking result about MDA was found in root-MDA in the application of 25 mM boric acid at low temperature. This result suggests that 10 mM boric acid application in the root helps to tolerate cold stress, but 25 mM boric acid application further increases the oxidative stress caused by low temperature (Fig. 1b).

In recent years, due to global climate change, plants are exposed to cold stress as much as they are exposed to sudden changes in temperature and high temperatures. As with other stress factors, plants have formed a complex antioxidant ROS scavenging system, including many non-enzymatic and enzymatic defense systems, to counteract the effects of low temperature. (Sato et al., 2001; Zhou et al., 2019; Fan et al., 2014).

In studies on green barley and strawberry leaves in the literature, it has been observed that the SOD, CAT and APX activities of plants exposed to cold stress increase (Yong et al., 2008; Radyuk et al., 2009; Jamshidi-Goharrizi et al., 2021)

There are studies in the literature on the effects of different abiotic stresses on different plant species. Tavallali et al. (2018)'s study on pistachio revealed that the boron concentrations that were applied had a positive effect in the plant's coping with salt stress by increasing SOD, CAT and APX activities. Therefore, it was pointed out that a sufficient source of boron increases the capacity of plant cells to tolerate oxidative stress (Tavallali et al., 2018). The results of our study supported this situation. In experiments conducted on maize, beans and wheat, it was reported that the combined effect of low temperature and salt stress was higher than the sole effect of each stress source in antioxidant enzyme activities (Demir, 2019). But no study was encountered in the literature to investigate the combined effects of low temperature and boron application.

It was determined that the combined effect of low temperature and boric acid increased the root-SOD and leaf-SOD activity and decreased root-APX, leaf-CAT, and leaf-APX activity compared to the optimum temperature (Figs 2a, 2c, 3a–3c).

Depending on the type of stress and the duration of application, the expression of antioxidative genes sometimes increases and sometimes decreases (Sato et al., 2001; Fan et al., 2014; Zhou et al., 2019). In our results, root-*SOD-Mn*, leaf-*SOD-Mn*, leaf-*SOD-Cu/Zn*, leaf-*CAT,* and leaf-*APX* gene expressions were decreased, and root-*APX* and leaf-*SOD-Fe* gene expressions were increased in plant samples treated with both low temperature and boric acid (Figs. 3–6a–c and 7–9). No study revealing the combined effects of low temperature and boron stress on enzyme gene expressions was encountered in the literature. However, there are studies where the combined effects of low-temperature stress and different abiotic stresses were examined. In these studies, it was reported that the synergistic effect of low-temperature stress with another stress was greater than the effect of low-temperature stress alone, which is consistent with our results (Liu et al., 2016; Zhou et al., 2017).

5. Conclusion

In this study, the effects of low and high temperatures and boron as a micronutrient on the sunflower plant were presented by investigating phenological parameters, antioxidant enzymes, and changes in the gene expressions of these enzymes. This study is thought to be the first to investigate the combined effect of low and high temperatures and two different boric acid concentrations. It was determined that antioxidant enzymes have a role in plants toleration of stress based on the increase in the enzyme activities and the mRNA levels of these enzymes. It was revealed that, with the effect of the single or combined applications of both high and low temperatures and boron, plant development was negatively affected, and as a reaction to these stresses, the antioxidant enzyme activities and their gene expressions increased. It may be stated that sunflower, which is a long-day plant, may be grown also in regions with low temperatures by supplementation of boron into the soil to a certain extent. It was determined that the combined effect of temperature and boron increased the damage on the plant, and boron application created more negative effects especially at a high temperature like 40 °C. However, at low temperatures, plant growth and yield increase may be supported by boron application. Therefore, studying multiple stress factors together proved to be more effective in understanding the actual life of plants in nature. Further studies may investigate changes occurring in the expressions of other genes by boron intake at extreme temperature, as well as the molecular responses created by combined stress factors in plants. Additionally, the mechanism of why boron created a positive effect on the plants at a low temperature and that of the damage created by boron at a high temperature should be examined.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This article produced from the master thesis of Fadime Donbaloğlu Bozca.

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