GAMMA-AMINO BUTYRIC ACID METABOLISM UNDER HIGH TEMPERATURE STRESS IN TWO LICHEN SPECIES

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Abstract. High temperature stress is a major environmental stress factor for all photosynthetic organisms. Some lichen species could have the ability of tolerance against global warming. In the present study, we investigated the effects of high temperature on GABA metabolism in two different lichen species *Evernia prunastri* and *Usnea* sp.. *Evernia* and *Usnea* sp. were collected from unpolluted locations in Bilecik, TURKEY. *Evernia* and *Usnea* sp. were kept at 45 °C for 0, 24 and 48 h. We analyzed GABA content, glutamate dehydrogenase (GDH) and glutamate decarboxylase (GAD) activities and also chlorophyll and MDA contents in the thalli of the lichens. The chlorophyll degradation and lipid peroxidation data indicated that *E. prunastri* thalli showed tolerance to high temperature while *Usnea* sp. thalli were found to be sensitive under these conditions. GABA content was enhanced by high temperature stress in *E. prutastri* thalli, while GAD and GDH activities were decreased. According to our results, we can suggest that GABA accumulation in lichen thalli could occur via different metabolic pathways.
Keywords: *Evernia, GABA, glutamate decarboxylase, glutamate dehydogenase, high temperature, Usnea*

Abbreviations: GABA: gamma-amino butyric acid; GAD: glutamate decarboxylase; GDH: glutamate dehydrogenase; MDA: malondialdehyde

Introduction

Global warming is one of the most serious problems for all organisms, populations, and ecological communities (Walther et al., 2002; Liu et al., 2003; Pisani et al., 2007). Climatic changes can negatively influence photosynthetic organisms; disrupt cellular homeostasis, and alter their morphology, biochemical structure, and physiology (Karl et al., 2007; Bita and Greats, 2013). High temperature stress can cause membrane damages, inhibit protein and carbohydrate metabolisms via inactivation of the enzymes in mitochondria and chloroplasts (Bita and Greats, 2013).

Gamma-amino butyric acid (GABA) plays different roles in plant metabolism including carbon–nitrogen metabolism, energy balance, signaling and development. It is found in a wide range of organisms including plants, algae and lichens (Bouché and Fromm, 2004;

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Yolcu et al., 2013). GABA probably plays a dual role as both a signaling molecule and a metabolite (Fait et al., 2008). GABA is mainly synthesized in metabolic pathway known as GABA shunt. It plays a major role in primary C/N metabolism. GABA shunt includes three main enzymes; cytosolic enzyme glutamate decarboxylase (GAD; EC 4.1.1.15), the mitochondrial enzymes GABA transaminase (GABA-T; EC 2.6.1.19), and succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.16). Enhancement in GABA content under various stress conditions such as drought, chilling, salt, heavy metal stresses was reported by several researchers (Bor et al., 2009; Al-Quraan et al., 2013; Wang et al., 2016). Previous studies demonstrated that GABA shunt pathway plays an important role in the alleviation of oxidative stress caused by high temperature stress (Cao et al., 2013; Nayyar et al., 2014).

Lichens are poikilohydric organisms and highly adapted to osmotic dehydration (Hájek et al., 2006). Most of the species have ability of tolerance against global warming and can live in the range of 35-46 °C and survive even at 70 °C (Pisani et al., 2007; Dyakov et al., 2015), although some air-dried lichen species are being sensitive to high temperature stress (Van Herk et al., 2002). Similarly, in higher plants, high temperature could have an effect on the growth and physiological process of the lichens (Pissani et al., 2007). Previously studies demonstrated that increasing temperature caused to a reduction in chlorophyll content and directly or indirectly altered the synthesis of lichen substances (Bjerke et al., 2003). In the literature, there are very limited studies on the heat tolerance or stress, and physiological mechanisms of high temperature tolerance are not fully known in lichens. Moreover, the role of GABA metabolic pathway under high temperature conditions is not clearly elucidated yet. In the present study, our aim was to determine the role of GABA shunt under high temperature conditions. To find out the answers of these questions, we showed that the alteration of GABA accumulation, and activities of GAD and GDH enzymes in different lichen species; *E. prunastri* and *Usnea* sp. under high temperature.

Materials and methods

Materials

The lichen species, *E. prunastri* and *Usnea* sp. were collected from unpolluted locations from Bilecik Center Forest, Turkey (N 40° 11.526', E 029° 57.962') at February 2017. 90 individual samples were transferred to the laboratory in the plastic bags, cleaned from impurities, and washed three times for 5 s with distilled water to remove dust from the surface. All lichen species were transferred to petri dishes and incubated in a growth chamber under 100 µmol m^{-2} s^{-1} light intensity and 16:8 light/dark cycle at 23 ± 2 °C and humidity was %38 for 48 h (Hall et al., 2002). Lichens were sprayed with distilled water once a day according to Calatayud et al. (1997). After laboratory adaptation, experimental conditions were designed. Lichen thalli was used as a control after the incubation for 48 h in normal growth conditions (as seen in *Table 1;* 0 h data).

For temperature experiments, 30 individual thalli of *E. prunastri* and *Usnea* sp. in petri dishes were kept for 0, 24 and 48 h in an incubator 100 µmol m^{-2} s^{-1} light intensity and 16:8 light/dark cycle at 45 ± 2 °C and likewise watered once a day. Heat stress temperature was chosen according to Pissani et al (2007) studies. The data presented in this study are the averages of three independent experiments.

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Table 1. *Chlorophyll a, and b content and Chlorophyll a/b ratio in thalli of E. prunastri and Usnea sp. under heat stress for 0 (as a control), 24 and 48 h*

Groups	n	*E. prunastri*			*Usnea* sp.		
		Chl*a* **(µg/mg fw)**	**Chl*b*** **(µg/mg fw)**	**Chl*a/b*** **(µg/mg fw)**	**Chl*a*** **(µg/mg fw)**	**Chl*b*** **(µg/mg fw)**	**Chl*a/b*** **(µg/mg fw)**
0 h	3	1.385±0.11	0.444±0.09	3.12±0.03	1.456±0.33	0.455±0.13	3.20±0.03
24 h	3	1.132±0.16	0.481±0.04	2.39±0.01	0.884±0.76	0.424±0.40	2.09±0.02
48 h	3	0.867±0.20	0.308±0.03	2.87±0.13	1.089±0.19	0.713±0.06	1.49±0.01
p-value		<0.0001	<0.0001	0.0005	0.0003	0.0002	<0.0001
F probability		802.63	291085.45	92.12	115.73	133.25	6600.25

Chlorophyll content analysis

Chlorophyll analysis was determined using the method described by Wellburn (1994). Approximately 20 mg of thalli were extracted in the dark for 1 h at 65 °C in 3.0 mL of dimethyl sulfoxide (DMSO) in the presence of polyvinylpolyrrolidone to minimise chlorophyll degradation. The absorbances of the extracts were measured using a UV-visible spectrophotometer (Perkin Elmer). For the chlorophyll analysis, measurements were taken at the wavelengths 665.1 and 649.1 nm.

Lipid peroxidation

Formation of malondialdehyde (MDA) was evaluated as an indicator of lipid peroxidation. The determination of MDA was performed by the thiobarbituric acid reactive substances method (Heath and Packer, 1968). The absorbance differences between 532 and 600 nm was used to calculate MDA formation as a by-product of lipid peroxidation.

GABA analysis

GABA content was detected by HPLC (Agilent 1200) according to Bor et al. (2009). 0.1 g of lichen samples (*E. prunastri* and *Usnea* sp. were homogenized in 5 mL solution of water:chloroform:methanol (3:5:12 v/v/v), and centrifuged at 4 °C for 10 min. The supernatant was dried. The dried samples were dissolved in water, Borax buffer (pH 8.0) and 2-hydroxynaphthaldehyde (0.3% in methanol) and heated at 80 °C for 30 min. The samples were separated by reversed-phase column Supelco LC18 (250 × 4.6 mm^2, 5 µm) at 330 nm with a mobile phase of methanol:water (62:38 v/v). The injection volume was 5 µL. GABA content was calculated according to the peak areas of GABA standarts.

GAD and GDH enzyme analysis

GDH activity was measured according to Akihiro et al. (2008) and Yolcu et al. (2013). The extracts were mixed with the assay mixture containing 50 mM $(NH_4)_2SO_4$, 13 mM α-ketoglutarate, 0.25 mM NADPH and 1 mM $CaCl_2$ in 100 mM Tris-HCl buffer (pH 8.0). GDH activity was measured according to the absorbance differences at 340 nm and expressed as micromoles per minute per gram fresh weight (FW).

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GAD activity was assayed according to Bartyzel et al. (2003) and Yolcu et al. (2013). 100 µL of sample was mixed with reaction mixture containing 3 mM L-glutamate, 20 µM pyridoxal phosphate and 50 mM potassium phosphate (pH 5.8) and incubated at 30 °C for 1 h for the decarboxylation process. HCl (0.5 M) was added to the mixture to stop the reaction. The samples were derivatized with 0.35% (w/v) ninhydrin solution. GAD activity was determined spectrophotometrically by comparing with the standard GABA values, and calculated as micromoles per minute per gram.

Statistics

Statistical analysis was performed with one-way analysis of variance (ANOVA) and Student's t-test followed by *posthoc* Tukey test as appropriate (SPSS for Windows version 11.0). The critical values for statistical significance was $p < 0.05$ and 0.01.

Results

Malondialdehyde (MDA) is often used as an indicator of oxidative damage at the cellular level. *Figure 1* shows the MDA content of thalli after exposure to high temperature for 0, 24 and 48 h. In *E. prunastri* thallus, MDA content was not significantly changed at 24 h, but the MDA content significantly increased ($p < 0.001$) at 48 h under high temperature (*Fig. 1*). In *Usnea* sp. thallus, the MDA content significantly increased at 24 h and 48 h.

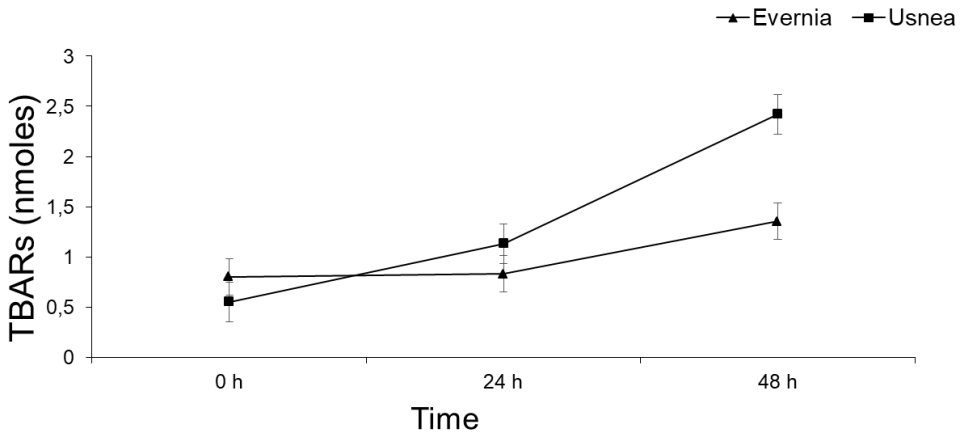

Figure 1. *Lipid peroxidation of different two lichen species under heat stress (45 °C) for 0 h, 24 h and 48 h*

The results of this study showed that the chlorophyll *a* content was significantly ($p < 0.001$) altered by high temperature treatment at 24 h and 48 h between lichen species. In *E. prunastri* thalli, however the chlorophyll *a* content gradually decreased at 24 and 48 h, chlorophyll *b* content was not significantly changed at 24 h (*Table 1*). The chlorophyll *a/b* ratio in the thallus of *E. prunastri* showed differences under 24 and 48 h exposures. In *Usnea* sp. The chlorophyll a content was significantly decreased at 24 and 48 h when compared to the control groups ($p < 0.001$) (0 h, as seen in *Table 1*). The content of chlorophyll *b* increased significantly under 48 h high temperature exposure. The chlorophyll *a/b* ratio decreased significantly after 24 h and 48 h exposures in *Usnea* sp. (*Table 1*).

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As shown in *Figure 2*, GABA was enhanced under heat especially by time exposures. The highest increase was determined in *E. prunastri* under 48 h heat treatment. The lowest increase was detected in *Usnea* sp. However, we did not find any significant increase in the GABA content under 24 h treatment in *Usnea* sp.

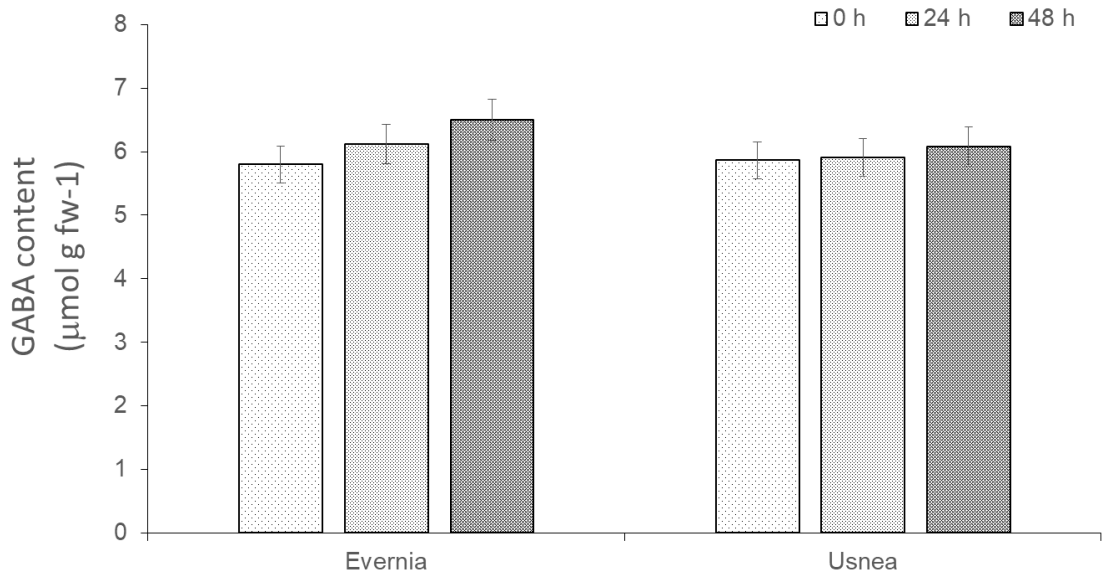

Figure 2. *GABA content of different two lichen species under heat stress (45 °C) for 0 h, 24 h and 48 h*

GAD activity was significantly decreased by heat treatments in *E. prunastri* when compared to the control group (*Fig. 3*). However, it was significantly increased by 24 h heat treatment in *Usnea* sp. as compared to the control group ($p < 0.05$). 48 h heat treatment did cause a decline in GAD activity in all of the species.

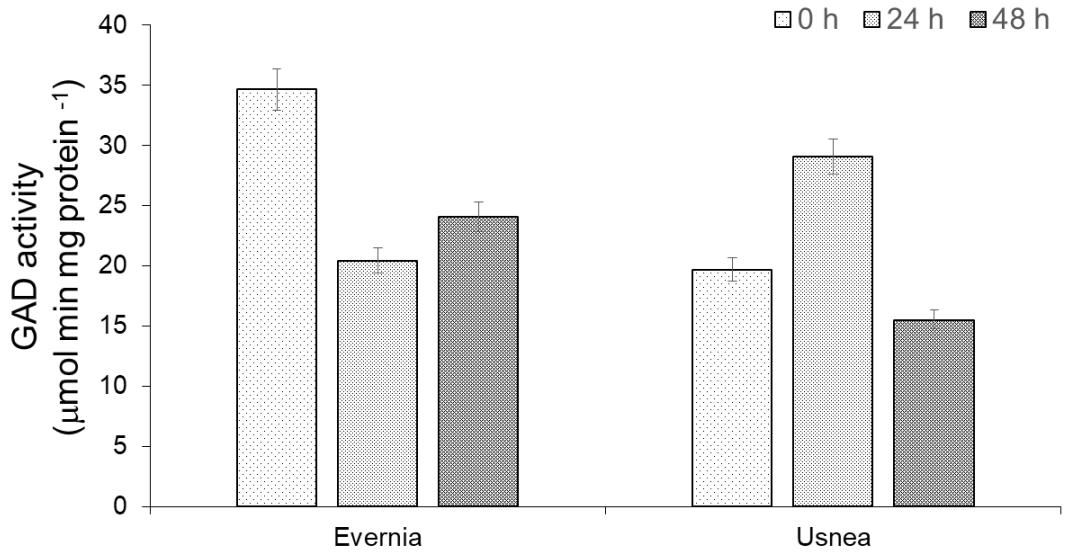

Figure 3. *GAD enzyme activities of different two lichen species under heat stress (45 °C) for 0 h, 24 h and 48 h*

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GDH activity was not significantly changed in both lichen species as compared to their control groups. However, GDH activity was increased under 24 h heat treatment in *E. prunastri* when compared to the control group (*Fig. 4*). In addition, GDH activity was significantly increased when compared to 48 h heat teratment in the thalli of *Usnea* sp ($p < 0.05$).

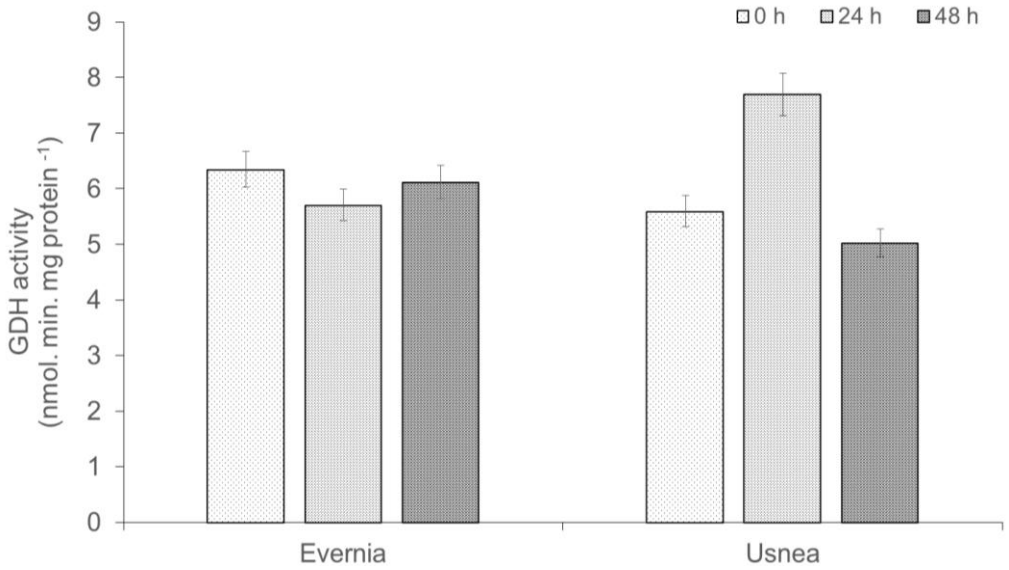

Figure 4. *GDH enzyme activities of different two lichen species under heat stress (45 °C) for 0 h, 24 h and 48 h*

Discussion

High temperature can induce oxidative stress via ROS production and lead can cause loss of cellular homeostasis in the photosynthetic organisms (Liu et al., 2003; Almeselmani et al., 2006). Oxidative stress also causes destruction of cell membranes via lipid peroxidation (Almeselmani et al., 2006). Malondialdehyde, MDA, is produced naturally as a result of lipid peroxidation and is often used as an indicator of oxidative damage at the cellular level (Mittler, 2002). Previous studies reported that high membrane damage occurs at higher temperatures and this could cause to the loss of structural organization of the membranes (Liu et al., 2003; Almeselmani et al., 2009; Hafmann, 2012). In our study, lipid peroxidation rate increased in *Usnea* sp., while MDA content did not significantly change in *E. prunastri* thalli at 24 h (*Fig. 1*). Our results indicate that thallus of *E. prunastri* could be more tolerant to heat stress.

Chlorophyll content is an important indicator for the photosynthetic process. It is also very sensitive to stress-initiated oxidative stress such as photo-oxidation (Chettri et al., 1998). Chlorophyll *b* is formed from chlorophyll *a* by the oxidation of the methyl group on the ring II to the aldehyde group (Chettri et al., 1998), and the ratio of chlorophyll *a/b* is more sensitive to the modification than chlorophyll *a+b*. In green plants, the antenna size is determined by the amount of light-harvesting chlorophyll *a/b* protein complex that is associated with the PSII (Takana and Takana, 2006). Conversion of chlorophyll *b* to chlorophyll *a* is the first step in chlorophyll degradation, as well as influencing chlorophyll a / b ratio (Chettri et al., 1998; Takana and Takana, 2006). Therefore, chlorophyll degradation is frequently used to identify the physiological

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conditions of the lichens (Chettri et al., 1998; Bačkor and Zetiková, 2003). Many lichen species could have tolerance to climate change and high temperature (Pisani et al., 2007). Pisani et al. (2007) showed that the content of photosynthetic pigments was not significantly changed at 40 °C for 24 h in *E. prunastri*. Similarly, in our study, chlorophyll degradation was not decreased in the range of 4-2 in *E. prunastri* thalli under high temperature stress (*Table 1*). However, the chl *a/b* ratio significantly decreased in the thalli of *Usnea sp.* during 24 and 48 h. Exposure of *Usnea* sp. to the temperature of 45 °C had significantly negative effect on the content of photosynthetic pigments (*Table 1*). Our results indicated that *E. prunastri* has more tolerance capacity than *Usnea* sp. under high temperature stress.

GABA biosynthesis is enhanced via various stress conditions (Bor et al., 2009; Al-Quraan et al., 2011; Paradisone et al., 2015), and GABA pathway could regulate the adaptation mechanisms (Al-Quraan and Al-Share, 2016). GABA is synthesized from glutamate by the activity of cytosolic GAD (Bouche et al., 2003). In higher plants, many researchers have suggested that GAD activity played a dominant role in the accumulation of GABA (Cao et al., 2012). Yolcu et al. (2013) also reported that GAD activity is highly correlated with high GABA levels. GDH is an enzyme found in the mitochondria that catalyzes the deamination of glutamate which is then converted to GABA by the action of GAD (Andersson and Rager, 2003; Forde and Lea, 2007). In higher plants, GABA accumulation can have a role in the tolerance under many environmental stress conditions (Nayyar et al., 2014). Previous studies also demonstrated that rapid accumulation of GABA in response to heat-stress (Bouche et al., 2004; Nayyar et al., 2014). In the present study, high GABA accumulation could decrease lipid peroxidation at 48 h with increasing lipid peroxidation rate at 48 h exposure in *E. prunastri* thalli (*Figs. 1* and *2*). Therefore, we can suggest that GABA could be a signal for high temperature tolerance.

Among lichens, Jager and Weigel (1978) reported that GABA is one of the predominant amino acids in the lichen *P. furfuracea*. Yolcu et al. (2013) also found that lowest GDH activity showed in *P. furfuracea* thalli as compared to higher plant species. In *Usnea* sp. under 24 h heat treatment, we can suggest that GABA could be synthesized by the activity of GAD via GABA pathway. However; under 48 h heat treatment GAD activity did not increase when compared to control group while GABA contents were enhanced under the same conditions (*Fig. 3*). In *E. prunastri* thalli, GDH and GAD enzymes activities did not show any relation with GABA accumulation under high temperature stress (*Figs. 3* and *4*). Numerous publications in higher plants link GABA metabolism with polyamine biosynthesis pathways, however there is not any information in lichens. GABA can also be synthesized via polyamine pathway by polyamine degradation or reduction (Moschou et al., 2008; Yolcu et al., 2013). Moreover, Wang et al. (2016) showed that the elevated chilling tolerance in NO-treated banana fruit may be attributed to enhanced accumulation of polyamines and GABA.

Conclusion

According to our results, we can suggest that GABA could be mainly synthesized via GAD activity during normal conditions in *Evernia* and *Usnea*, but in stress conditions, GABA can be synthesized via polyamine accumulation. However, further studies should be done to identify the effects of polyamine pathway on GABA metabolism in lichens both under normal and stress conditions.

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Author contributions. All authors conceived research. Dr. Dilek Unal designed the experimental framework and chlorophyll and lipid peroxidation analysis. Dr. Fazilet Ozlem Cekic performed GABA and enzyme analyses. Dr. Nihal Goren-Saglam, Dr. Emel Yigit and Dr. Hulya Torun participated the experimental analysis. Dr. Dilek Unal and Dr. Nihal Goren-Saglam drafted the manuscript. All authors have read and approved the manuscript.

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