

The Role of Gamma-Amino Butyric Acid in Short-Term High Temperature Acclimation in Lichen *Pseudevernia furfuracea*

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ABSTRACT

Objective: Global warming causes many different stresses for plants. High-temperature stress is one of the important problems caused by global warming. Plants develop various tolerance mechanisms to protect themselves against these stresses. γ -Aminobutyric acid (GABA) metabolism has a critical role in various biological processes in plants. GABA plays a critical role in the acclimation to different stress conditions in plants. Lichens can grow in environments exposed to severe abiotic stresses such as drought and extreme heat. The major aim of this study was to identify whether GABA accumulation could improve short-term, high-temperature tolerance in lichen *Pseudevernia furfuracea*.

Materials and Methods: For this aim, *P. furfuracea* samples were kept in petri dishes in an incubator at $45 \pm 2^\circ\text{C}$ for 24 and 48 h. We analyzed the chlorophyll a/b ratio, GABA content, glutamate decarboxylase (GAD) and glutamate dehydrogenase (GDH) activities, which are important enzymes involved in the GABA shunt, and also peroxidase (POD) and catalase (CAT) activities of the antioxidant metabolism.

Results: Our study indicated that the chlorophyll a/b ratio was not changed significantly under 45°C within 48 h. POD and CAT activities were significantly decreased in lichen thalli under 45°C , however; GABA accumulation was approximately enhanced by 1.5-fold depending on the time exposure. GAD and GDH activities were significantly increased under high temperature conditions.

Conclusions: The acclimation of *P. furfuracea* to high temperatures may be related to the increase in GAD and GDH activities. Our findings provided evidence that the GABA shunt could help lichen *P. furfuracea* to acclimate to high temperatures.

Keywords: Acclimation, antioxidant mechanism, GABA, high temperature, lichen, *Pseudevernia furfuracea*

INTRODUCTION

Many researchers suggest that increases in global temperature will be the biggest problem in the future (1,2). Understanding the factors that regulate high temperatures and tolerance mechanisms which belong to different organisms are very important subjects for the scientific community. Besides, lichens are symbiotic organisms and have a high adaptation capacity to various environmental conditions including high temperatures

(2,3). Previous studies demonstrated that some lichen species have remarkable thermotolerance and they can survive between $35\text{--}46^\circ\text{C}$ (2,3). It is known that lichens include first- and second-line protection systems such as producing phytochelatins, lichen acids, non-thiol compounds and activated antioxidant systems under different stress conditions, especially metal toxicity (4). The antioxidant defense mechanism, including enzymatic and non-enzymatic systems, is a fundamental reactive oxygen scavenger system to deal with oxidative stress



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in fungi, animal and plant systems, as well as lichen. In higher plants and lichens, antioxidant capacity determines the ability of plants to acclimate or survive against high temperatures (4-6). In addition, γ -aminobutyric acid (GABA) metabolism could be included in tolerance mechanisms to high temperatures (7-9). However, the physiological mechanisms of thermotolerance in lichens have not been clearly understood yet.

GABA has critical roles in the acclimation to different stress conditions in plants and animals (10-12). Previous researches have also shown that GABA could have important roles in the metabolism of plants such as amino acid metabolism, nitrogen repartition, and the signalling and development processes due to the close link to the Tricarboxylic acid cycle (13,14). Besides, GABA biosynthesis occurs in animals, plants, algae and lichens (14,15). Glutamate decarboxylase (GAD; EC 4.1.1.15), GABA transaminase (GABA-T; EC 2.6.1.19), and succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.16) have important roles in the metabolic pathway of GABA. Recent studies have indicated that GAD participates in the regulation of metabolism and can play a considerable role in signal transduction under abiotic stress (15,16). Although an increase in content of GABA by abiotic stress factors such as high temperatures, chilling, drought, salt and heavy metal stresses were shown in the previous studies (8,17-20), GABA metabolism in lichens under different abiotic stress conditions, especially high temperature, is not well known. We tried to figure out the function of GABA metabolism and antioxidant enzyme activity in lichens exposed to high temperature acclimation. For this purpose, we indicated the activities of GAD and GDH and the change in GABA accumulation in *Pseudevernia furfuracea*.

MATERIALS AND METHODS

Plant Materials

P. furfuracea (including *Trebouxia*, green algae as photobiont) were carefully collected from an unpolluted location in Bilecik Center Forest, Turkey (N 40° 11.526', E 029° 57.962'). The collected lichen thalli were cleaned and washed with dH₂O until their surfaces were cleared of dust. All of the lichen thalli were incubated at 23 ± 2°C, 16 h light/8 h under 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity for 48 h in a growth cabinet. Experimental conditions were designed according to the laboratory environment. *P. furfuracea* samples were kept in petri dishes in an incubator (100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity, 16 h light/8 h dark, 45 ± 2°C) for 24 and 48 h. The lichen thallus was watered once a day. The data presented are means of three independent experiments. Each treatment was comprised of three replicates.

Chlorophyll Content Analysis

The lichen thalli (20 mg) were extracted in the dark in 3.0 ml dimethyl sulfoxide (DMSO) with polyvinylpyrrolidone for 1 h at 65°C to minimize the chlorophyll degradation (21). The chlorophyll content was measured at 665.1 and 649.1 nm.

Analysis of GABA Content

Measurement of GABA content was performed via HPLC (Agilent 1200) (18). The lichen samples (0.1 g of *P. furfuracea*) were

homogenized in a water:chloroform:methanol solution, and then centrifuged for 10 min at 4°C. After that, and we dried the supernatants and then dissolved them in an ultra-pure H₂O, borax buffer solution with 2-hydroxynaphthaldehyde and then incubated at 80°C for 30 min. We analyzed the samples at 330 nm by reversed-phase column (250x4.6 mm², 5 μm , Supelco LC18) and the mobile phase was methanol:water (62:38 ratio). While calculating, GABA standard peak areas were taken into consideration.

GAD and GDH Analysis

The lichen extracts were mixed with a reaction mixture ((NH₄)₂SO₄, α -ketoglutarate, NADPH and CaCl₂ in Tris-HCl buffer). The activity of GDH was analyzed spectrophotometrically at 340 nm according to Akihiro et al. (22) and Yolcu et al. (14).

GAD activity was measured by Bartyzel et al. (17) and Yolcu et al. (14). 100 μL of sample was added to an assay mixture (L-glutamate, pyridoxal phosphate and potassium phosphate), mixed, and for the decarboxylation process, we kept it at 30°C for 1 h. To stop the reaction we added HCl to the mixture. Then we centrifuged the mixture for 10 min at 12,500 x g. All of the samples were derivatized with ninhydrin solution. We calculated GAD activity by comparison with the GABA standards.

MDA Content

We analyzed malondialdehyde (MDA) content by the TBA (thio-barbituric acid) method (23). For the calculation of the MDA content, we used the differences in absorbances at 532 and 600 nm.

Antioxidant Enzyme Analysis

20 mg of the thalli were homogenized in liquid nitrogen with pestle and mortar. We resuspended the pellet with 4 mL of homogenization buffer (50 mM phosphate buffer, 1 mM EDTA, 2% TritonX-100 and 2% polyvinylpyrrolidone, pH 7.5). The pellet continued to homogenize with pestle and mortar. Afterwards, the sample was centrifuged at 12,000 g for 20 min at 4°C and then transferred to new Eppendorf tubes. Obtained total protein extracts used as the enzyme extract immediately and kept on ice during experiments.

Catalase (CAT) (EC 1.11.1.6) activity was analyzed in the reaction mixture (100 mM phosphate buffer, the extract and 6 mM H₂O₂). The breakdown of H₂O₂ was measured at 240 nm. Calculation of the CAT activity was made according to Bergmeyer (24) by using an extinction coefficient of 39.4 mM⁻¹ cm⁻¹.

The ascorbate peroxidase (APX) (EC 1.11.1.11) activity was analyzed at 290 nm in accordance with the decrease in the absorbance of the substrate (25). The oxidized ascorbate's concentration was calculated by using an extinction coefficient of 2.8 mM⁻¹ cm⁻¹. One unit of APX activity was described as the $\mu\text{mol mL}^{-1}$ oxidized ascorbate per min and APX content was defined as unit. mg⁻¹ total soluble protein.

The peroxidase (POD) (EC 1.11.1.7) activity was analyzed spectrophotometrically at 470 nm according to (26). The reaction

solution was formed by mixing the guaiacol (0.25%) in a sodium phosphate buffer (0.1 mol/L, pH 7.0, 0.1% hydrogen peroxide). The crude enzyme extract (60 μ L) was added to initiate the reaction, which is based on the oxidation of guaiacol. POD activity was defined as $\Delta A/g\text{-Fr}\cdot W\cdot x\text{Min}$.

The protein amount was measured according to the Bradford method (27).

Statistical Analysis

All the data obtained in the study were subjected to one-way ANOVA and we also used Pearson's correlation test. The comparisons between ($p<0.01$ and $p<0.05$) were regarded as significantly different.

RESULTS

GABA accumulation was increased under heat treatments with time exposures (Figure 1). It was found that the highest increase in GABA content was in *P. furfuracea* after 48 h heat application ($p<0.001$). In this study, the activities of GAD and GDH were determined in order to understand whether GABA was enhanced in the lichen thalli. The activity of GAD was increased under heat treatments *P. furfuracea* compared to the control group, significantly (Figure 2a). In *P. furfuracea*, GAD activity increased approximately by 1.5-fold under high temperatures at 48 h. Similarly, GDH activity significantly increased in comparison with the control group ($p<0.05$, Figure 2b). GDH

activity of *P. furfuracea* thalli at 45°C increased approximately 1.4- and 1.3-fold respectively, in the 24 h comparison with the control ($P<0.05$).

We found that chlorophyll a and chlorophyll a+b contents were significantly decreased under high temperatures at 48 h (Table 1). However, the chlorophyll a/b ratio was not markedly changed under high temperatures at 24 and 48 h.

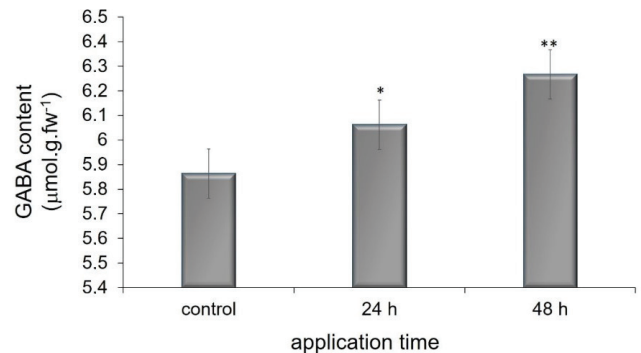


Figure 1. The GABA content of *P. furfuracea* exposed to high temperature (45°C) for 0 (as a control), 24 and 48 h.*Represents a statistically significant difference of $p<0.05$ when compared with the control, **represents a statistically significant difference of $p<0.01$ when compared with the control.

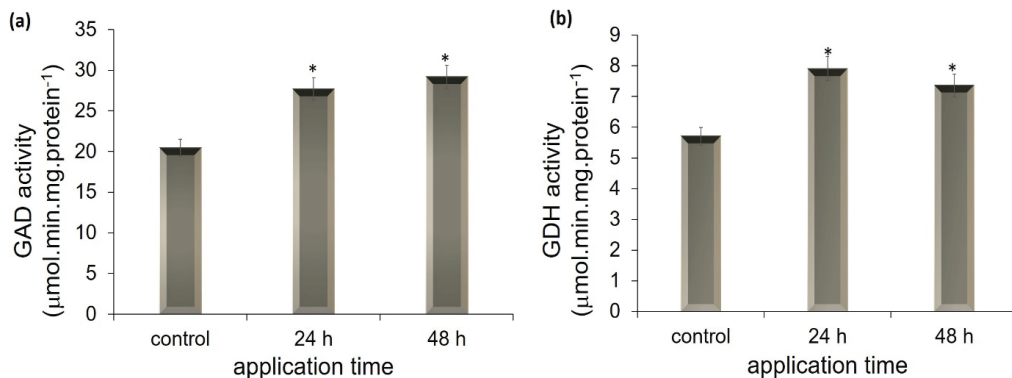


Figure 2. GABA biosynthesis enzymes activities in *P. furfuracea* exposed to high temperature (45°C) for 0 (as a control), 24 and 48 h. (a) GAD activity; (b) GDH activity *Represents a statistically significant difference of $p<0.05$ when compared with the control.

Table 1. Chlorophyll a, b and a+b content and Chlorophyll a/b ratio in thalli of *P. furfuracea* under high temperature for 0 (as a control), 24 and 48 h.

Groups	n	Chla ($\mu\text{g}/\text{mg fw}$)	Chlb ($\mu\text{g}/\text{mg fw}$)	Chla+b ($\mu\text{g}/\text{mg fw}$)	Chla/b ($\mu\text{g}/\text{mg fw}$)
Control (0 h)	3	1.58 $\pm$ 0.02	0.516 $\pm$ 0.04	2.096 $\pm$ 0.02	3.06 $\pm$ 0.02
24 h	3	1.518 $\pm$ 0.04	0.502 $\pm$ 0.06	2.023 $\pm$ 0.03	3.02 $\pm$ 0.04
48 h	3	1.471$\pm$0.03*	0.478 $\pm$ 0.06	1.949$\pm$0.03*	3.08 $\pm$ 0.06

*Represents a statistically significant difference of $p<0.05$ when compared with the control.

Figure 3 indicates the MDA content after the high-temperature treatment for 0, 24 and 48 h exposure. In the thallus of *P. furfuracea*, the MDA content was increased significantly ($p < 0.05$) at 24 and 48 h under high temperatures (Figure 3).

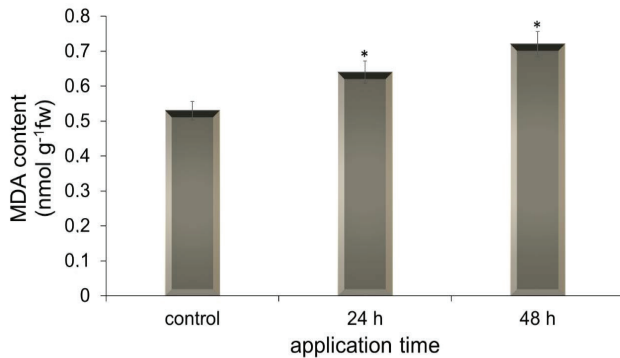


Figure 3. Malondialdehyde (MDA) content of *P. furfuracea* exposed to high temperature (45°C) for 0 (as a control), 24 and 48 h. *Represents a statistically significant difference of $p < 0.05$ when compared with the control

The effects of high temperatures on the enzyme activities of *P. furfuracea* is shown in Figures 4a–c. As shown in Figures 4a and b, the POD and CAT activities gradually decreased due to high temperatures at 24 and 48 h. However, APX activity of *P. furfuracea* was 1.51-fold higher than the control group at 24 h ($p < 0.05$, Figure 4c). Besides, APX activity of *P. furfuracea* was significantly reduced at 48 h comparison with the control group ($p < 0.05$).

Pearson's correlation results indicated that lipid peroxidation rate as an oxidative stress marker was related to the GABA metabolic pathway (Table 2). MDA level was strongly positively correlated with GABA content and GAD activity (Table 2). In contrast, GDH activity indicated a moderate positive correlation with lipid peroxidation. MDA content also had a strongly positive correlation with CAT activity. In addition, the antioxidant enzyme activity, especially POD activity, showed a strong negative correlation with GAD activity. POD activity also had a strong positive correlation with CAT and APX activities (Table 2).

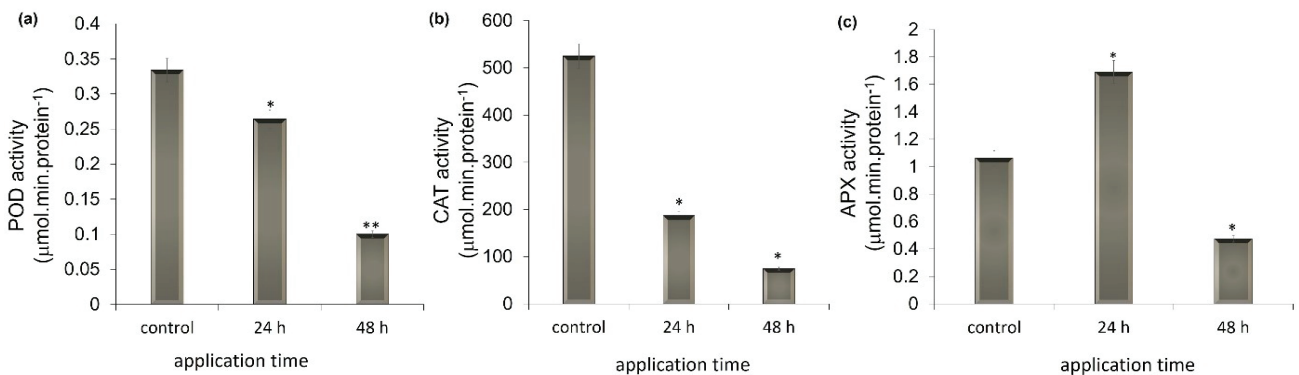


Figure 4. The antioxidant enzymes activities of *P. furfuracea* exposed to high temperature (45°C) for 0 (as a control), 24 and 48 h. (a) peroxidase (POD) activity; (b) catalase (CAT) activity; (c) ascorbate peroxidase (APX) activity. *Represents a statistically significant difference of $p < 0.05$ when compared with the control, **represents a statistically significant difference of $p < 0.01$ when compared with the control.

Table 2. Pearson's correlation test results (R^2 value) about physiological parameters under high temperature. * $p < 0.05$, ** $p < 0.01$

	MDA	GABA	GDH	GAD	POD	APX	CAT
MDA	-	0.9231**	0.5129	0.7439*	-0.963**	-0.6192	-0.8376**
GABA	0.9231**	-	0.5583	0.8877**	-0.4009	-0.4423	-0.8848**
GDH	0.5129	0.5583	-	0.7138*	-0.5114	0.1109	-0.702
GAD	0.7439*	0.8877**	0.7138*	-	-0.6722*	-0.1648	-0.8449**
POD	-0.963**	-0.4009	-0.5114	-0.6722*	-	0.6739*	0.8171**
APX	-0.6192	-0.4423	0.1109	-0.1648	0.6739*	-	0.1826
CAT	-0.8376**	-0.8848**	-0.702*	-0.8449**	0.8171**	0.1826	-

DISCUSSION

High temperatures negatively affect photosynthetic organisms via inhibition of photosynthesis, distribution of cell membrane integrity and structure, though it causes damage to or inhibits many biological processes such as carbon metabolism (5,8,28). Chlorophyll is also a molecule sensitive to altered environmental conditions and chlorophyll content is frequently used for the determination of physiological conditions of lichen thalli (29-32). Many lichen researchers have demonstrated that chlorophyll a/b ratio is a more sensitive marker than chlorophyll a+b value (29,30). Previous studies have reported that high temperatures cause decreased chlorophyll content in higher plants (8,9). However, chlorophyll content of some lichen species was not significantly changed under high temperatures (2,32). In the present study, chlorophyll a+b content significantly decreased in *P. furfuracea* thalli at 48 h. In contrast, the chlorophyll a/b ratio was not significantly changed in *P. furfuracea* thalli at 45°C for 24 and 48 h. These results suggest that the photobiont of *P. furfuracea* could acclimate to high temperatures.

GABA metabolism could play a remarkable role for acclimation to high temperatures (7-9). Our previous work demonstrated that GABA content increased under high temperatures in *Evernia prunastri* thalli and this increase could be related to high-temperature tolerance (32). Similarly, in the present study, GABA accumulation was observed under high-temperature condition (Figure 1).

In higher plants, GABA is mainly produced from glutamate via cytosolic GAD enzyme activity (16,33). For glutamate to convert to GABA, glutamate needs deamination from the mitochondrial GDH enzyme (34,35). Yolcu et al. (14) demonstrated that *P. furfuracea* had the lowest GDH activity under normal conditions. In the present study, the GAD and GDH enzyme activities increased under high-temperature stress (Figure 2). These results demonstrated that GABA accumulation could occur in a short time and it could be synthesized via GAD activity as in higher plants. In yeast and higher plants, rapid GABA acclimation also occurred in response to heat stress (7,8,20). Similarly, Sadowsky et al. (36) reported that GABA content was increased during desiccation in the strains of green algal photobionts (*Trebouxia*). In both photosynthetic and non-photosynthetic regimes, TCA-related amino acids could have important roles in various metabolic processes (37). Yolcu et al. (14) also indicated that in *P. furfuracea* GABA could be one of the prepotent amino acids, which is in agreement with our findings. Moreover, the enzymes of GABA shunt, especially GAD activity, has a critical role in reducing the effect of oxidative stress (20,38). In our study, GAD could have also a special role under high-temperature stress.

Lipid peroxidation rate is a good indicator for understanding oxidative damage in cells. In the present study, the lipid peroxidation rate increased depending on the time of exposure to high temperatures (Figure 3). Interestingly, lipid peroxidation rate and GAD activity showed a positive correlation (Table 2). While in plants the role of GABA as a signal molecule has been proven,

in lichens, further studies should be done to identify the role of GABA metabolism. Moreover, GABA content in different parts of plants could indicate the impacts of atmospheric pollution (39), and different parts of the lichens should be well evaluated.

Antioxidant mechanisms provide for the scavenging of reactive oxygen species (ROS) and acclimation to environmental stress conditions (5,9,38). Previous studies have demonstrated that high temperatures caused a decrease in CAT and POD activities (9) and increased APX activity (8,9). Similarly, in this study, APX activity enhanced within 24 h, while CAT and POD activities decreased (Figure 4). In addition, there is much information in the literature that exogenous GABA may enhance CAT, POD and APX activities under different stress conditions in higher plants (8,40). However Li et al. (9) demonstrated that the application of GABA did not stimulate CAT and POD activities. In our study, CAT and POD activities indicated a negative correlation with GABA accumulation and its biosynthetic enzymes (Table 2). Moreover, APX activity did not show any positive correlation with GABA metabolism. On the other hand, increased APX activity in 24 h could result in acclimation to high temperatures.

CONCLUSION

In summary, physiological analysis of thalli of *P. furfuracea* under short time high temperatures demonstrated that GABA could improve high-temperature acclimation through multiple physiological processes including protection of photosynthesis, and the improvement of some antioxidant enzyme activities. Lichens have a high tolerance to climate change, and the increase in GABA content may trigger some metabolic pathways in the mycobiont or photobiont sides. Our results demonstrated that GABA metabolism could have a role in lichen acclimating to temperature stress. However, future research should focus on the role of GABA shunt as a stress signaling molecule on different metabolic pathways in lichen under short- and long-term different stress conditions.

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