Turkish Journal of Biology

Volume 35 | Number 1 Article 7

1-1-2011

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Turk J Biol
35 (2011) 59-68

doi:10.3906/biy-0904-21

# Is the induction of $H_2O_2$-detoxifying antioxidant enzyme activities sufficient to protect barley cultivars from oxidative stress by UV-B irradiation alone or pretreatment with high temperature and NaCl?

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Received: 30.04.2009

**Abstract:** Ultraviolet B (UV-B) irradiation has an adverse effect on plant cells because it causes the generation of reactive oxygen species (ROS). This is scavenged by some endogenous defense systems. In this study, the activities of $H_2O_2$-detoxifying antioxidant enzymes in 4 barley cultivars pretreated with high temperature or NaCl before UV-B irradiation were investigated. The stress response in the seedlings was determined by measuring the photosynthetic performance index (PI), which significantly decreased in all barley cultivars under almost all treatments compared to their controls. Activities of 3 $H_2O_2$-detoxifying antioxidant enzymes (ascorbate peroxidase, glutathione reductase, and peroxidase) significantly increased as a result of all treatments. The results may suggest that UV-B irradiation induces major $H_2O_2$-detoxifying enzyme activities, and pretreatments, especially with 200 mM of NaCl, enhance the response of enzyme activities in these 4 barley cultivars. Changes in the PI responses could indicate that the induction of antioxidant enzymes might not be enough to protect from oxidative stress induced by UV-B radiation in the barley cultivars investigated in the study.

**Key words:** Ascorbate peroxidase, barley, glutathione reductase, high temperature, NaCl, peroxidase, photosynthetic performance index, UV-B irradiation

## Arpa çeşitlerinin yalnız veya yüksek sıcaklık ve NaCl ön uygulamalı UV-B ışımasıyla oluşan oksidatif stresten korunmak için $H_2O_2$ detoksifiye eden antioksidan enzim aktivitelerinin indüksiyonu yeterli midir?

**Özet:** Ultra viyole-B ışıması, reaktif oksijen türleri (ROS) oluşturması nedeni ile bitki hücrelerinde olumsuz bir etkiye sahiptir. Bu oluşum bazı içsel savunma sistemleri ile ortadan kaldırılmaktadır. Çalışmada, UV-B ışımasından önce yüksek sıcaklık ve NaCl ön uygulamasına maruz bırakılan dört arpa çeşidinde $H_2O_2$ detoksifikiye eden antioksidan enzimlerin aktiviteleri araştırılmıştır. Fidelerdeki stres zararı, fotosentetik performans indeksi (PI) ölçümleriyle belirlenmiştir ve PI tüm arpa çeşitlerinde hemen hemen tüm uygulamalarda önemli derecede azalmıştır. PI'nin aksine, çalışmada araştırılan üç $H_2O_2$ detoksifiye eden antioksidan enzimlerin (askorbat peroksidaz, glutatyon redüktaz ve peroksidaz) aktiviteleri genellikle tüm uygulamalarda önemli düzeyde artmıştır. Elde edilen sonuçlar, UV-B ışımasının önemli $H_2O_2$ detoksifiye eden enzimleri indüklediğini ve özellikle 200 mM NaCl olmak üzere, ön uygulamalar bu dört arpa çeşidinde cevabı artırdığını ortaya koyabilir. Ancak PI cevaplarına göre, $H_2O_2$ detoksifiye eden antioksidan enzimlerin indüksiyonunun çalışmada araştırılan arpa çeşitlerinde UV-B ışımasının teşvik ettiği oksidatif stresten korunmak için muhtemelen yeterli olmadığı sonucuna varılabilir.

**Anahtar sözcükler:** Askorbat peroksidaz, arpa, glutatyon redüktaz, yüksek sıcaklık, NaCl, peroksidaz, fotosentetik performans indeks, UV-B radyasyonu

## Introduction

Continuous climate change may induce several stress factors, such as drought, high temperature, salinity, and enhanced UV-B radiation, simultaneously (1,2). In nature, the plant encounters stress combinations concurrently or at different times through the growing season (3) and must present an integrated response to them (4). Consequently, the acclimation of plants to a combination of different abiotic stresses would require an appropriate response customized to each of the individual stress conditions involved, as well as tailored to the need to compensate or adjust for some of the antagonistic aspects of the stress combination (5). Mittler (6) has suggested that it is logical to assume that the simultaneous exposure of a plant to different abiotic stress conditions will result in the coactivation of different stress response pathways. These might have a synergistic or antagonistic effect on each other. In addition to that, there are cases where the plant organism subjected to a single stress agent is capable of increasing its resistance to subsequent unfavorable impacts (7), which is called cross-acclimation. For example, heat stress was found to silence the UV-B response of parsley (8), whereas salt pretreatment mitigates UV-B adverse effects in barley (9-12).

It has been claimed that the amount of solar ultraviolet-B radiation (UV-B, 280-320 nm) at the earth's surface has been increasing because of stratospheric ozone depletion (1,13). Responses to UV-B radiation exhibit variations in the higher plant species (14). It has been reported that UV-B radiation can alter the redox state of plants through the increased production of reactive oxygen species (ROS) (15-18), causing oxidative stress in cells.

A major part of the injury to plants exposed to stress is related to oxidative damage at the cellular level (19). Although under normal metabolic processes the low amounts of ROS are metabolic by-products of plant cells (20,21), the release of radicals into the cytosol can be enhanced under certain stress conditions (22), producing oxidative stress in cells. $O_2^{\bullet-}$ radicals are immediately converted into $O_2$ and hydrogen peroxide ($H_2O_2$). Therefore, production of these radicals results in an increase of $H_2O_2$ in the cell (22). The generation of $H_2O_2$ is increased in response to various stresses, implicating it as a key factor mediating the phenomena of acclimation and cross-tolerance, in which previous exposure to one stress can induce tolerance of subsequent exposure to the same or different stresses (23,24).

Although the precise intracellular concentrations of $H_2O_2$ that are likely to be toxic will vary, high rates of $H_2O_2$ production are normally balanced by very efficient antioxidant systems (25). If there is a serious imbalance in any cell compartment between the production of ROS and antioxidant defense, oxidative stress and damage occur (6). Abiotic stresses such as dehydration, low and high temperatures, and excess irradiation can disturb this balance in such a way that increased $H_2O_2$ initiates signaling responses, including enzyme activation, gene expression, programmed cell death, and cellular damage.

Plants have developed ROS scavenging systems, which are categorized as enzymatic and nonenzymatic (26,27). When ROS increases, the chain reactions start, i.e. superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot-}$ radicals to molecular $O_2$ and $H_2O_2$ (28). $H_2O_2$ is then detoxified in the ascorbate-glutathione cycle (6,29), which involves the oxidation and rereduction of ascorbate and glutathione through the APX and GR action (25).

The activities of antioxidant enzymes like SOD, APX, and GR are enhanced by treatment with UV-B in some plants (30,31), and salt-tolerant plant species increased their antioxidant enzyme activities and antioxidant contents in response to salt treatment (32).

The objective of this study was to clarify the effect of pretreatment with high temperature (45 °C for 45 min) or NaCl (200 mM) before UV-B irradiation on the $H_2O_2$-detoxifying antioxidant enzymes responses in 4 barley cultivars at the seedling stage and to determine whether there would be cross-acclimation to UV-B.

## Materials and methods

### Plant materials

The seedlings of 4 barley (*Hordeum vulgare* L.) cultivars (Bülbül-89, Kalaycı-97, Tarm-92, and Tokak-157/37) were used in this study. They were grown in the southern and southwest regions of Turkey, where the climate is warmer.

**UV-B treatment**

UV-B was artificially provided by UV-B (312 ± 25 nm) fluorescent tubes (G15T8E, USHIO). The distance between the top of the plants and the UV-B lamp was 30 cm. The lamp irradiation gave a photon flux density of 2.88 kJ $m^{-2}$ $day^{-1}$. During UV-B treatment, no white light was applied. The biological effectiveness of UV-B radiation ($UV\text{-}B_{BE}$) was calculated using the plant action spectrum of Caldwell (33), normalized to unity at 300 nm.

**High temperature pretreatment**

Plants were grown in a controlled growth chamber at 25/20 ± 0.2 °C day/night temperature, with a 16 h photoperiod under fluorescent white light (200 µmol $m^{-2}$ $s^{-1}$ PPFD) and a relative humidity of 60 ± 5%. Six-day-old plants were subjected to 45 °C for 45 min. After 24 h, the seedlings were exposed to UV-B irradiation for 5 h ($UV\text{-}B_1$).

**Salt pretreatment**

Plants were grown in water culture in a growth chamber as described in the previous section. Two-day-old plants were supplied with 200 mM NaCl for 4 days, which was enough to induce salt stress in these barley cultivars (12). The seedlings were then exposed to UV-B for 1 h on each of 2 consecutive days ($UV\text{-}B_2$).

Sampling for measurements was conducted 24 h after UV-B treatment for the 2 experiments.

**Chlorophyll *a* fluorescence measurements**

Fluorescence measurements were made at room temperature with a Handy-PEA fluorometer (Hansatech Instruments Ltd., King's Lynn, Norfolk, UK). Dark-adapted leaves (at least 60 min) were illuminated homogeneously over an area of 4 mm in diameter with an array of 3 red LEDs (3000 µmol photons $m^{-2}$ $s^{-1}$), and chlorophyll *a* fluorescence signals were received by a high performance PIN photodiode detector associated with an amplifier circuit. Based on the theory of energy fluxes in biomembranes in a photosynthetic sample, the performance index on an absorption basis, $PI_{abs}$, has been calculated using the experimental values of the polyphasic rise of chlorophyll fluorescence transients provided from the JIP-test (34,35). The performance index (PI) is one of the chlorophyll fluorescence parameters that provide useful and quantitative information about the state of plants and their vitality (36). After the fluorescence measurements, the leaves were harvested to use for the measurements of enzyme activities.

**Assays of antioxidant enzyme activities**

Leaves (0.3 g) from control and treated plants were ground with liquid nitrogen and suspended in specific buffer and pH for each enzyme extraction. The homogenates were centrifuged at 14,000 rpm for 20 min at 4 °C, and the resulting supernatants were used for enzyme assay. The protein concentrations of the leaf crude extract were determined according to the method of Bradford (37).

The APX activity was determined according to the method of Wang et al. (38). APX extraction was performed in a suspension solution including 50 mM Tris-HCl (pH 7.2), 2% PVP, 1 mM $Na_2EDTA$, and 2 mM ascorbate. The assay solution contained 50 mM potassium phosphate buffer (pH 6.6), 2.5 mM ascorbate, 10 mM $H_2O_2$, and an enzyme extract containing 100 µg of protein. The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient of ascorbate (ε = 2.8 mM $cm^{-1}$ at 290 nm).

The GR activity was assayed following the method of Sgherri et al. (39). GR extraction was performed in a suspension solution containing 100 mM potassium phosphate buffer (pH 7.0), 1 mM $Na_2EDTA$, and 2% PVP. The assay mixture contained 200 mM potassium phosphate buffer (pH 7.5), 0.2 mM $Na_2EDTA$, 1.5 mM $MgCl_2$, 0.5 mM GSSG, 50 µM NADPH, and an enzyme extract containing 100 µg of protein. Correction was made for the nonenzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to the assay mixture. The enzyme activity was calculated from the initial rate of the reaction after subtracting the nonenzymatic oxidation using the extinction coefficient of NADPH (ε = 6.2 mM $cm^{-1}$ at 340 nm).

The POD activity was based on the determination of guaiacol oxidation (ε = extinction coefficient 26.6 mM $cm^{-1}$) at 470 nm by $H_2O_2$. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 20.1 mM guaiacol, 12.3 mM $H_2O_2$, and 100 µL of enzyme extract (40).

The SOD activity was assayed according to the method of Beyer and Fridovich (41). The reaction mixture of 30.25 mL was composed of 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 μM nitroblue tetrazolium (NBT), and an appropriate volume of the plant extract. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibited NBT reduction.

### Statistical analysis

The experiment was performed using a randomized block design. Data presented are the averages of at least 3 replicates, obtained from 3 independent experiments. SPSS was used to establish the differences between the cultivars and treatments. Statistical variance analysis of the data was performed using ANOVA and compared with the least significant differences (LSD) at the 5% level.

## Results and discussion

### Photosynthetic performance index

Photosynthesis is a major process in plant physiology, and its functional state has been considered a significant physiological activity to assess the responses of plants to environmental parameters (42). The photosynthetic performance index (PI), giving information about the state of plants and their vitality, was seriously decreased by UV-B irradiation in all barley cultivars. The seedlings pretreated with high temperature (45 °C for 45 min) and NaCl (200 mM for 4 days) before UV-B irradiation also showed very low PI values (Table). The lowest decrease was determined in Tarm-92 under UV-$B_1$ and UV-$B_2$ radiation alone, whereas the highest decrease was in Tokak-157/37 under UV-$B_1$ and Kalaycı-97 under UV-$B_2$, compared to their controls. High temperature treatment alone decreased the PI by 9%, 17%, and 44% in the Bülbül-89, Tarm-92, and Tokak-157/37 cultivars, respectively, and increased the PI of Kalaycı-97 by 16% (Table). Pretreatment with high temperature significantly alleviated the adverse effect of UV-B on PI in Bülbül-89 and Kalaycı-97 compared to UV-$B_1$ radiation alone. This treatment aggravated the negative UV-B effect in Tarm-92 (Table). Salt treatment alone also decreased the PI of all cultivars, but to a lesser extent in comparison to UV-B and pretreatment with high temperature and salt (Table). UV-$B_2$ decreased the PI of all barley cultivars compared to their controls in NaCl pretreated plants (Table). Similar to high temperature pretreatment, salt pretreatment also aggravated the damaging effect of UV-B in Tarm-92. In the present research, UV-B treatment significantly affected photosynthetic performance. Decreased PI may indicate the imbalance of the electron transport chain in the photosynthetic process and suggest an oxidative stress formation in all cultivars under almost all treatments. In terms of photosynthetic performance, these results showed that pretreatment with 45 °C for 45 min or 200 mM NaCl for 4 days did not elicit any cross-tolerance to UV-B in the seedlings of the 4 barley cultivars studied.

### Antioxidant enzymes activities

It is known that UV-B irradiation causes oxidative stress (17,43-47). Xu et al. (47) reported that several

Table. Changes in photosynthetic performance index (PI)* of 4 barley cultivars exposed to UV-B alone or pre-treated with high temperature (45 °C for 45 min) and NaCl (200 mM for 4 days). UV-B was applied for 5 h only 1 day (UV-$B_1$) and 1 h for 2 consecutive days (UV-$B_2$) to the seedling treated with high temperature and NaCl, respectively.

| Cultivars | Treatments | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | UV-$B_1$ | 45 °C | 45 °C+UV-$B_1$ | UV-$B_2$ | NaCl | NaCl+UV-$B_2$ |
| Bülbül-89 | 1.000 | 0.0812 | 0.9087 | 0.2424 | 0.0849 | 0.6457 | 0.1179 |
| Kalaycı-97 | 1.000 | 0.0752 | 1.1643 | 0.1259 | 0.0594 | 0.4087 | 0.0691 |
| Tarm-92 | 1.000 | 0.1183 | 0.8278 | 0.0223 | 0.1385 | 0.8049 | 0.0726 |
| Tokak-157/37 | 1.000 | 0.0514 | 0.5587 | 0.0597 | 0.0657 | 0.4955 | 0.0564 |

* Data were normalized by the value of their own control plants for every barley cultivar using Biolyzer software.

antioxidant pools and activities of several key enzymes involved in ROS metabolism were affected by UV-B. In the present study, UV-B generally increased antioxidant enzyme activities, and pretreatments with high temperature or salt enhanced the effect of UV-B on the investigated enzymes in all cultivars (Figures 1-3). In addition, superoxide dismutase activity (SOD) was measured according to the method of Beyer and Fridovich (41), but its activity was not changed by UV-B (data not shown). Our findings were consistent with the data of Mazza et al. (15), which determined that UV-B did not affect the SOD activity in barley. Rao et al. (30) also indicated that UV-B exposure preferentially induces peroxidase-related enzymes instead of SOD. Therefore, we focused on $H_2O_2$-detoxifying enzyme activity in the present study.

When subjected to UV-B radiation alone or pretreated with high temperature and salt, different responses in $H_2O_2$-detoxifying enzyme activities were observed in the 4 barley cultivars (Figures 1-3). APX that is one of the most important enzymes in the ascorbate-glutathione cycle, and $H_2O_2$ detoxification showed variations in the cultivars and treatments (Figure 1). UV-B treatment alone significantly increased the APX activity in all cultivars compared with their controls. The APX activities of Kalaycı-97 and Tokak-157/37 were slightly affected by high temperature treatment (Figure 1a), whereas the activities of Bülbül-89 and Tarm-92 were slightly

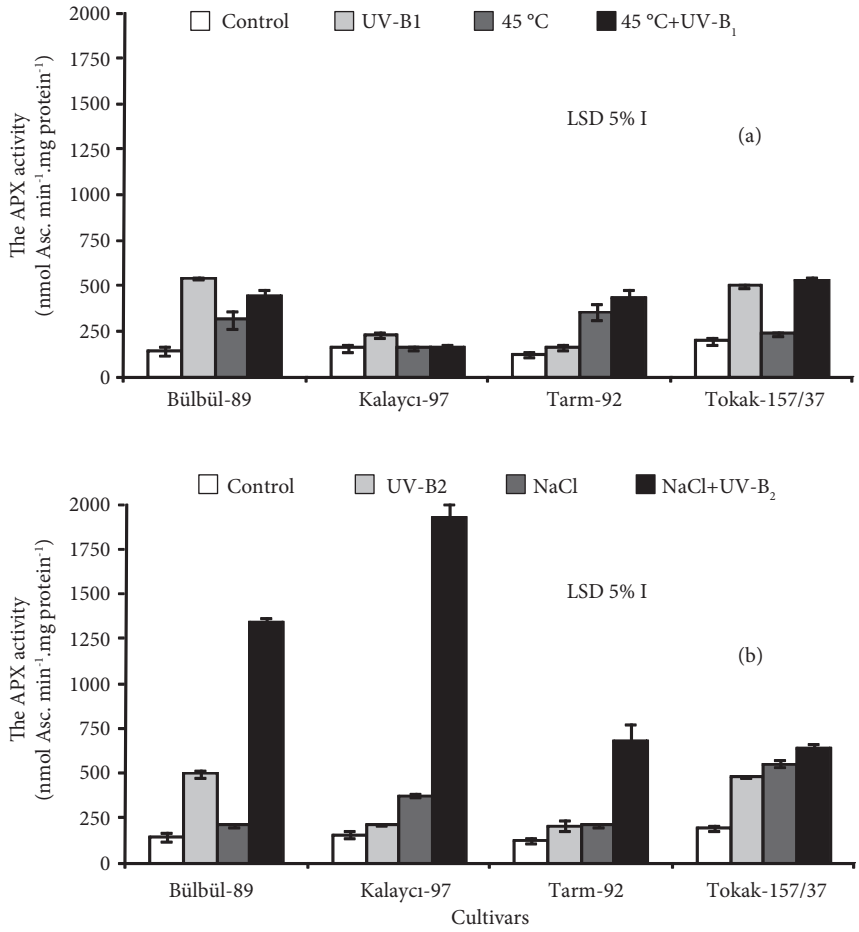


Figure 1. Effects of UV-B radiation on the APX activity in pre-treated with high temperature (a) and NaCl (b) of barley cultivars. See Table for explanation of UV-B application.

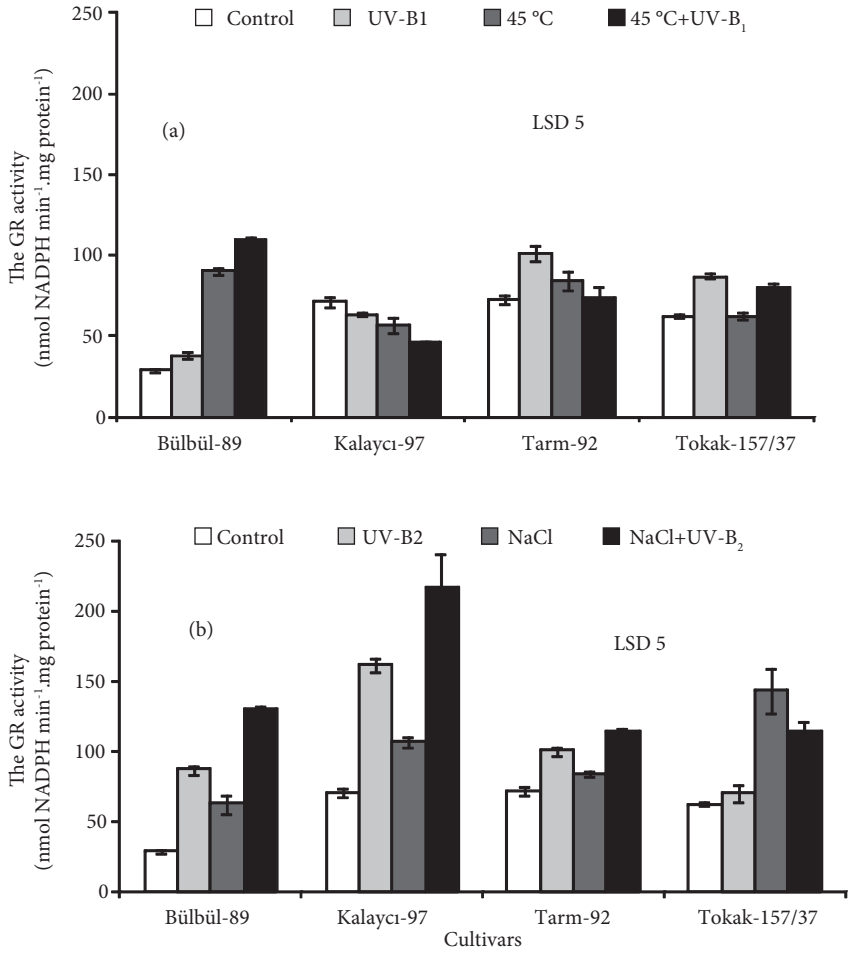


Figure 2. Effects of UV-B radiation on the GR activity in pre-treated with high temperature (a) and NaCl (b) of barley cultivars. See Table for explanation of UV-B application.

affected by salt treatment (Figure 1b). A temperature of 45 °C and UV-B treatment also increased the activity in all cultivars by approximately 3.0-3.6 times compared to the control, except Kalaycı-97 (Figure 1a). Salt and UV-B treatment increased the APX activity in the 4 barley cultivars (Figure 1b). Furthermore, the APX activities of Bülbül-89 and Kalaycı-97 were nearly 9.3 and 13.3 times higher than those of the controls, respectively. Pretreatment with NaCl before UV-B exposure enhanced APX activity much more than pretreatment with high temperature, especially in Kalaycı-97 and Bülbül-89 (Figures 1a and 1b). High temperature and salt alone or UV-B irradiation of pretreated cultivars with high temperature did not change the activity much. These results suggest that APX had an important role in the control of endogenous $H_2O_2$ content in the seedlings pretreated with NaCl before UV-B irradiation. According to some research (48,49), the stimulation of APX activity by salt stress was much higher in salt-tolerant cultivars. Our findings are also in agreement with Takeuchi et al. (50), who have found that UV-B enhanced APX activity in cucumber cotyledons.

The activity of glutathione reductase, the other enzyme of the ascorbate-glutathione cycle, was variable among both cultivars and treatments (Figure 2). UV-B treatment alone markedly increased the GR activity in all cultivars compared with the controls, except in

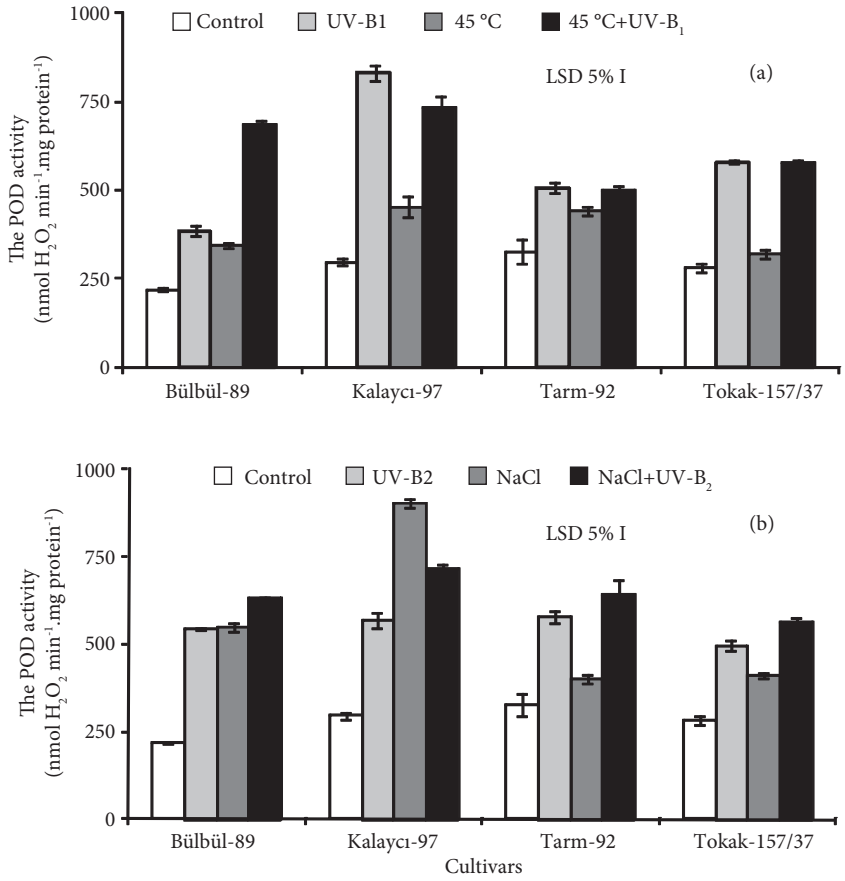


Figure 3. Effects of UV-B radiation on the POD activity in pre-treated with high temperature (a) and NaCl (b) of barley cultivars. See Table for explanation of UV-B application.

the Kalaycı-97 exposed to high temperature pretreatment. High temperature alone increased the GR activity of Bülbül-89 and Tarm-92 and decreased the GR activity of Kalaycı-97 (Figure 2a), while salt alone significantly increased the activity in all examined cultivars (Figure 2b). A temperature of 45 °C and UV-B treatment increased the activity in Bülbül-89 and Tokak-157/37, but decreased the activity in Kalaycı-97 and did not significantly change the activity in Tarm-92, compared with their controls (Figure 2a). Salt and UV-B treatment significantly increased the GR activity in the 4 barley cultivars (Figure 2b). The GR activity levels of the cultivars were approximately 4-7 folds higher than those of controls except for Tarm-92. Noctor and Foyer (25) reported that the major substrate for reductive detoxification of $H_2O_2$ via APX reaction, ascorbate, must be continuously regenerated from its oxidized forms via the ascorbate-glutathione cycle. Accordingly, GR activity was also increased by almost all treatments, except in Kalaycı-97 (Figure 2). This result may indicate that GR could work adequately and the ascorbate-glutathione cycle might be active in the investigated barley cultivars. It was shown that salt treatment had little effect on the activity of glutathione reductase (51), and it was suggested that its lower activity in the stressed roots could be due to some acclimation or an inability to maintain a high GSH/GSSG ratio (48,52).

The results of peroxidase activity, one of the $H_2O_2$ detoxification pathway enzymes, are presented in Figure 3. All treatments significantly increased the

POD activity of barley cultivars. The highest increase in the POD activity was found in the seedlings exposed to UV-B irradiation alone (UV-$B_1$ and UV-$B_2$) and pretreated with high temperature or salt, except for the Bülbül-89 and Kalaycı-97 cultivars in the salt experiment (Figures 3a and 3b).

It has been assumed that increased UV-tolerance correlates with increased peroxidase activity (53,54). If so, we could suggest that the salt pretreatment enhanced UV-B tolerance in the 4 barley cultivars more than the temperature pretreatment. In the present study, POD increased in all barley cultivars as a result of all treatments. POD activity was the highest in the plants pretreated with either high temperature (Figure 3a) or NaCl (Figure 3b) before UV-B irradiation. Results are consistent with those of Tekchandani and Guruprasad (55). Similarly, Hideg et al. (56) reported that reversible drought stress improved the tolerance of plants against subsequent UV-B irradiation and that the link between 2 stresses was at the level of ROS scavenging, possibly in the provocation of the antioxidant system by the preceding water stress.

Borisova et al. (57) proclaimed that the data of the interaction of excess heat and UV-B irradiation in plants were contradictory. They found that a preliminary heat treatment (45 °C, 1 h) considerably attenuated the adverse effect of UV-B irradiation. In the present study, heat pretreatment decreased APX and GR activities in Kalaycı-97 cultivars (Figures 1a and 2a) and had no effect on the other cultivars.

In conclusion, UV-B irradiation decreased photosynthetic performance, whereas it increased APX, GR, and POD activities in the seedlings of 4 barley cultivars. This is in accordance with the findings of Mishra et al. (58), which showed that the seedling stage is the most vulnerable stage of plant development. These results may imply that the induction of antioxidant enzyme activities might not be enough to protect the barley cultivars investigated in this study from oxidative stress caused by UV-B irradiation. The results of the present study show that pretreatment with high temperature (45°C, 45 min) did not provide an acclimation to UV-B, as NaCl did. Moreover, induction of the $H_2O_2$-detoxifying antioxidant enzyme activities of barley cultivars subjected to UV-B irradiation alone or after pretreatments cannot prevent UV-B stress due to increased ROS formation in the leaves. To clarify the mechanism of cross-acclimation as a result of pretreatment with high temperature or NaCl before UV-B irradiation, further investigations are needed on the physiological and biochemical responses and the recovery process.

**Acknowledgements**

The authors are grateful to Şeküre Çulha, Hacettepe University, for her assistance in the experiments. This research was supported by the Scientific and Technological Research Council of Turkey, project number TBAG-U/135, and the Bulgarian Academy of Sciences.

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