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DROUGHT AND EXCESS UV-B IRRADIATION DIFFERENTIALLY ALTER THE ANTIOXIDANT SYSTEM IN CUCUMBER LEAVES

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The combined effects of enhanced UV-B radiation and soil drought on antioxidant enzyme activity were investigated in cucumber leaves. One-month-old cucumber plants (*Cucumis sativus* cv. Dar) were exposed to UV-B irradiation and water deficit alone or combined. Physiological measurements were made in seedlings kept under stress conditions for nine days and then two more days with stresses withdrawn. Generally a decrease in relative water content and an increase in dry weight content were recorded. The more significant changes were observed under drought than under UV-B radiation and or combined UV-B and drought. Both stresses stimulated antioxidant enzyme activity. Superoxide dismutase activity increased earlier (day 2) than guaiacol peroxidase and glutathione reductase activity (days 5 and 7). Elevation of enzyme activities was higher under drought than under UV-B. Combined UV-B and drought functioned synergistically: one of the stresses reduced the effects caused by simultaneous application of the other.

Key words: Glutathione reductase, guaiacol peroxidase, soil drought, superoxide dismutase, ultraviolet-B.

INTRODUCTION

Almost all environmental stresses induce oxidative stress in plants as an early and rapid response (Bolkhina et al., 2003). Under drought stress, overproduction of highly reactive oxygen species (ROS) in chloroplasts, represented by the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^{1/2}O_2$) has been reported (Smirnoff, 2002; Foyer and Noctor, 2003). This effect was also observed in chloroplasts of plants under enhanced UV-B irradiation (Santos et al., 2004). Plants have an endogenous mechanism to protect cellular and subcellular systems from the cytotoxic effects of ROS. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), Halliwell-Asada pathway enzymes and peroxidases (POX) are widely distributed in all higher plants and are involved in decomposition of $O_2^{\cdot-}$ and H_2O_2 (Foyer and Noctor, 2000). Peroxidases, especially their anionic forms located in cell walls, are engaged in peroxidation of phenolic compounds in the presence of H_2O_2 (Grabber et al., 1997). These processes cause lignification of the cell wall, creating a barrier against pathogen attack (Lewis and Yamamoto, 1990), and also increasing the rigidity of the cell wall under drought conditions (DaMatta et al., 2002).

Enhanced UV-B radiation or drought can decrease net photosynthetic capacity and lead to the reduction of root, stem and leaf biomass and yield. Such effects were shown to be greater under a combination of the two stresses than under single ones (Feng et al., 2007). The results suggested that the co-stresses of supplementary UV-B irradiation and drought functioned synergistically, and that one of them could differentially affect the inhibitory effects of the other. Recent studies indicate that the response of a plant to a combination of two different abiotic stresses is unique and cannot be extrapolated from the response to each stress applied alone. Tolerance to a combination of stress conditions should be the focus of research aimed at developing plants with enhanced tolerance to naturally occurring environmental conditions. Surprisingly, the co-occurrence of different stresses is rarely addressed by molecular biologists, and its physiological aspects are also insufficiently understood (Mittler, 2006).

This study was intended to determine how two environmental stresses, water deficit and UV-B radiation, applied alone or together, can alter the activity of the antioxidant enzymes SOD, glutathione

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reductase (GR) and guaiacol peroxidase (GPX) in cucumber leaves. Also investigated was how these stresses influence some acclimatization changes within the cell walls of cucumber leaves, based on syringaldazine peroxidase (SPX) activity.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of cucumber (*Cucumis sativus* cv. Dar) were sown in a pot containing 2.0 l loessial soil and allowed to germinate and develop in a growth chamber with a 16 h photoperiod (250 μE $m^{-2} \cdot s^{-1}$ photosynthetically active radiation, PhAR) at 24/20°C (day/night) and 60–70% humidity. Water content in the soil was maintained at 60% of holding capacity. One-month-old seedlings were divided into four groups, the control and three groups subjected to the stress conditions: UV-B radiation, water deficit, and water deficit and UV-B radiation combined.

STRESSES

For the UV-B stress treatment, UV-B radiation was supplied by Philips TL 20 W/01 RS lamps at 16 KJ m^{-2} d^{-1} (8 h daily) for 9 days. The photon flux density was 3.25 μmol m^2 s^{-1} at 315 nm. For the water deficit treatment, water supply was restricted, reducing soil water content to 40% of holding capacity. The two stresses were applied, alone or combined, for 9 days, after which the plants were watered well and UV-B irradiation was ended in the respective treatment groups, and they were kept for two more days of post-stress physiological measurements. Control plants were watered well and kept under PhAR only. The third fully expanded leaf of each seedling was used for analysis. They were sampled at the outset of the experiment, on days 2, 5, 7 and 9 of the stress treatment, and on day 11.

RELATIVE WATER CONTENT (RWC)

Relative water content, indicating the level of water stress in leaves, was estimated according to Weatherley (1950) and calculated according to the formula: RWC = [(fresh weight – dry weight) / (fresh weight at full turgor – dry weight)] · 100%.

ENZYME ACTIVITY

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was estimated according to Beauchamp and Fridovich (1971). Leaves (200 mg) were homogenized with 4 mL (w/v) of 50 mM Na-phosphate buffer, pH 7.0, containing 1% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA-Na and 0.5 M (w/v) NaCl. The reaction mixture contained 50 mM Na-phosphate buffer, pH 7.8, 0.1 mM (w/v) EDTA-Na, 13 mM (w/v) methionine, 25 μM (w/v) nitro blue tetrazolium (NBT), 2.4 μM (w/v) riboflavin and 0.03 mL enzyme extract. The addition of riboflavin and the placement of tubes under fluorescent lamps ensuring irradiation intensity of 185 $\mu E \cdot m^{-2} \cdot s^{-1}$ started the reaction of blue formazan accumulation. The increase in optical density was almost linear for at least 10 min. Tubes without the enzyme developed maximum color. Absorbance at 560 nm was recorded and 1 unit of activity was estimated as the enzyme quantity reducing absorbance to 50% of the value for tubes lacking the enzyme.

Guaiacol peroxidase (GPX) (EC 1.11.1.7) measurement was based on the method of Hammerschmidt et al. (1982). Leaves were homogenized in ice-cold 50 mM K-phosphate buffer, pH 7.5, with the addition of 5% (w/v) polyvinylpolypyrrolidone (PVPP). Enzyme assays were prepared by the addition to a glass cuvette of 0.5 mL 50 mM K-phosphate buffer, pH 7.5, 0.5 mL extract, 0.5 mL 3.4 mM guaiacol and 0.5 mL 0.9 mM H_2O_2. Absorbance at 480 nm was measured and guaiacol oxidation was expressed as units per minute per mg protein. One unit of enzyme activity caused an increase of absorbance by 0.1 per min.

Glutathione reductase (GR) (EC 1.6.4.2) was measured using the method described by Smirnoff and Colombe (1988). Leaves were homogenized in 5 vols of ice-cold extractant. The extraction medium contained 100 mM K-phosphate buffer, pH 7.8, 1 mM diethylenetriamine penta-acetic acid (DTPA), 5% polyvinylpyrrolidone (PVP), 5 mM mercaptoethanol and 5% (v/v) glycerol. The homogenate was centrifuged at 4°C for 30 min at 25,000 g. The reaction mixture contained 50 mM K-phosphate buffer, pH 7.5, 0.1 mM NADPH, 0.5 mM oxidized glutathione and 100 μl extract per 1 ml reaction mixture. The rate of NADPH oxidation was monitored at 340 nm.

Syringaldazine peroxidase (SPX) (EC 1.11.1.7) was determined according to Imberty et al. (1985). Leaf samples (400 mg) were extracted in 100 mM K-phosphate buffer, pH 7.0, containing 0.5% polyethylene glycol (PEG 6.000) and 40 mg Polyclar AT. After centrifugation at 10,000 g for 15 min at 4°C, the extract (0.5 ml) was mixed with 2 ml 100 mM K-phosphate buffer, pH 6.0, 0.5 ml 4.0 mM H_2O_2, and 50 μl syringaldazine (3.1 mg dissolved in 1 ml methanol and mixed with 2 ml dioxane). Enzyme activity was calculated as absorbance increase at 530 nm.

PROTEIN CONTENT

Protein content in the extracts was determined according to Bradford (1976).

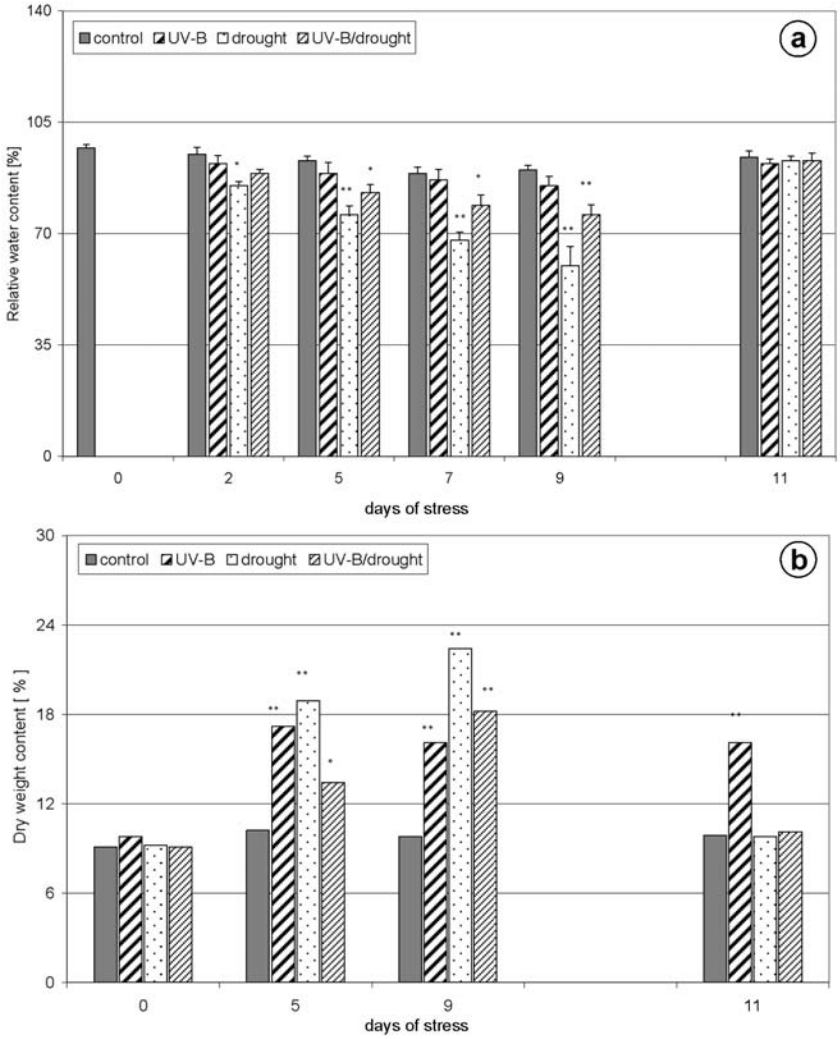

Fig. 1. Changes in relative water content (RWC) (**a**) and dry weight content (%) under progressive UV-B, water deficit and co-stresses in cucumber leaves (**b**). Measurements were made from days 0 to 9 of stress, and 2 days after stress treatment. Values are means ±SE (n = 3). Significance of differences between stressed and control plants: *p<0.05, **p<0.01.

STATISTICAL ANALYSIS

Analyses were done in replicates and the data are presented as means ±SD. Experimental data were subjected to one-way ANOVA and the significance of differences between means was determined by Tukey's multiple range test.

RESULTS

Water deficit greatly lowered the RWC of cucumber leaves, by as much as 60% at the end of the stress period (Fig. 1a). UV-B radiation decreased the leaf water content only to 85%, but both stresses together reduced it to 75% on the 9th day of stress. After rewatering or/and withholding excess UV-B radiation, the water content of leaves generally returned to the control level. In well-watered plants, RWC remained above 90% throughout the experimental period.

In the leaves of control plants, dry weight was 9–10% of fresh weight (Fig. 1b). Under stress, dehydration of plant tissues caused a significant increase in this parameter versus the control. The effect was more pronounced in the leaves of cucumber plants under water stress (19–22%) than under UV-B irradiation (16–17%) and under water stress/UV-B co-treatment (14–18%). In leaves under UV-B stress this effect persisted at almost the same level (16%) after recovery of RWC to over 90%.

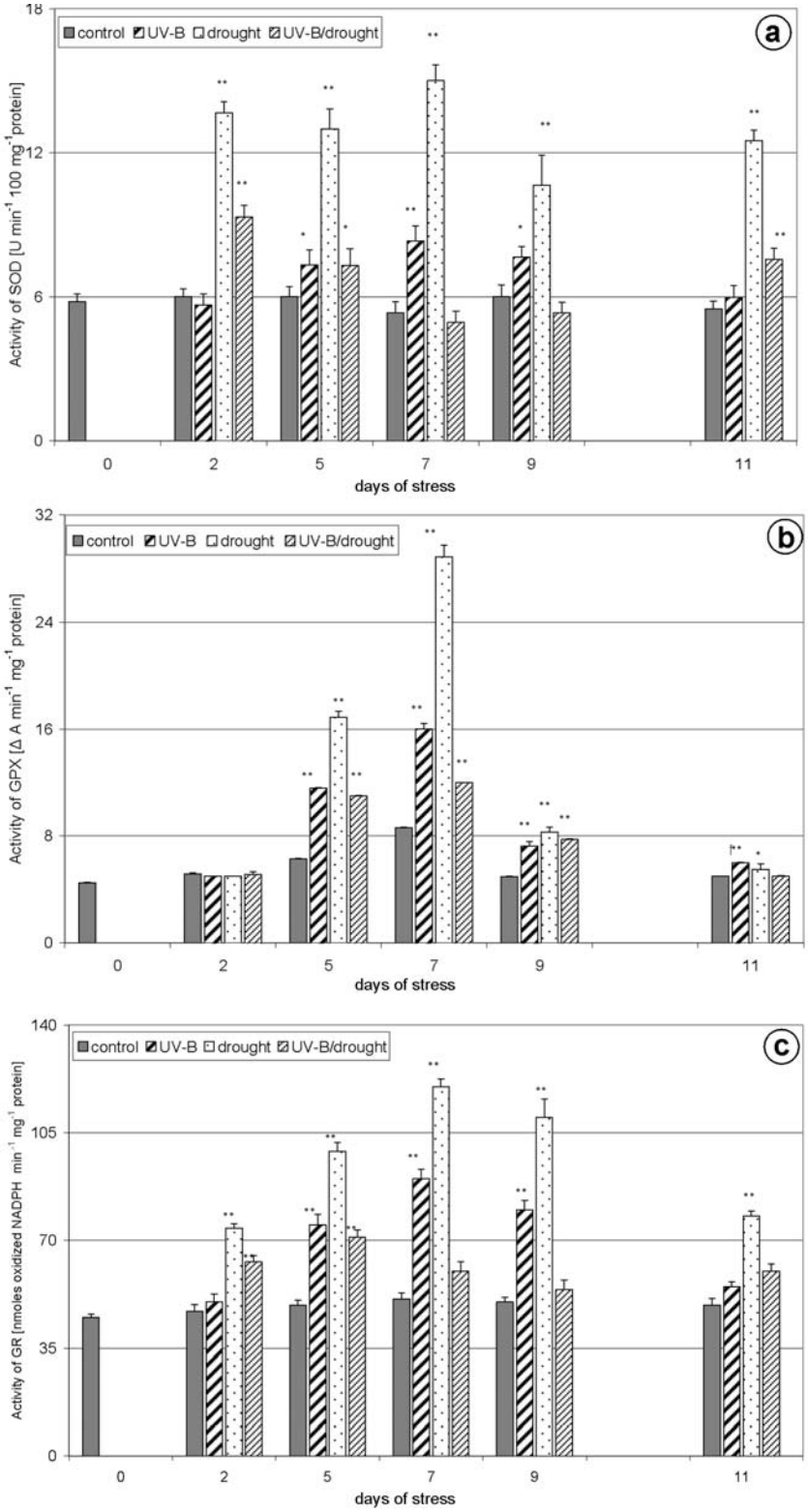

Fig. 2. Effect of progressive UV-B, water deficit and co-stresses on antioxidant enzyme activity in cucumber leaves. (**a**) Superoxide dismutase (SOD), (**b**) Guaiacol peroxidase (GPX), (**c**) Glutathione reductase (GR). Measurements were made from days 0 to 9 of stress, and 2 days after stress treatment. Values are means ±SE (n = 5). Significance of differences between stressed and control plants: *$p<0.05$, **$p<0.01$.

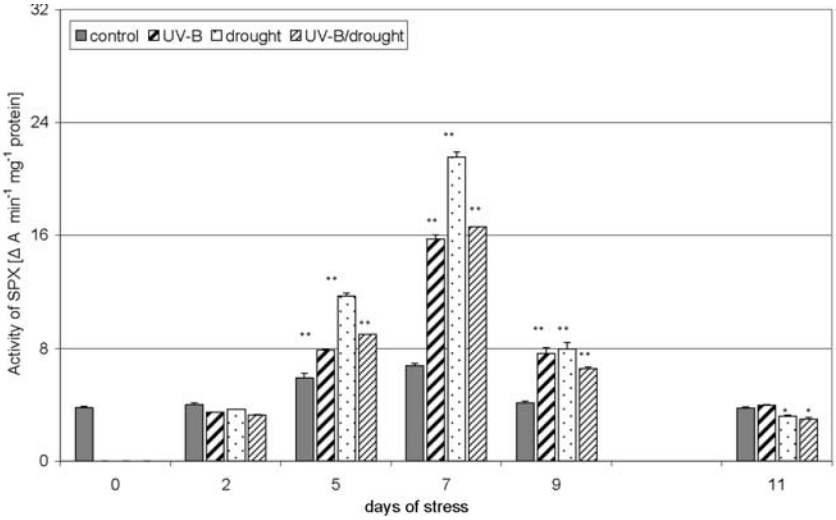

Fig. 3. Effect of progressive UV-B, water deficit and co-stresses on syringaldazine peroxidase (SPX) activity in cucumber leaves. Measurements were made from days 0 to 9 of stress, and 2 days after stress treatment. Values are means ±SE (n = 5). Significance of differences between stressed and control plants: *p<0.05, **p<0.01.

Stress-induced alteration of antioxidant enzymes was observed (Fig. 2): the highest (triple) increase was in GPX; SOD and GR activity doubled versus the control. SOD activity (Fig. 2a) greatly increased under drought stress on day 7. UV-B radiation did not cause such a significant increase on day 7. Under combined stresses, SOD activation was observed earlier, on day 2. In control plants, activity was stable throughout the experiment. The highest increase in GPX activity (Fig. 2b) was recorded on day 7, and in plants under water deficit it reached the highest level; on that day it was intermediate under UV-B radiation and lowest under drought and UV-B co-treatment. After rewatering and/or ending the UV-B treatment, GPX activity dropped to the control level. The highest increase in GR activity (Fig. 2c) also occurred on day 7 of drought; it was lower in plants under UV-B, and lowest (60) under combined drought and UV-B. After withdrawal of the stresses, GR activity decreased to the control level except in water-stressed plants, in which it remained slightly higher.

The applied stress factors altered the activity of syringaldazine peroxidase (Fig. 3). The highest increase versus the control was measured on day 7 of drought; it was lower after UV-B radiation and combined drought and UV-B. On day 11 of the experiment, that is, 2 days after withdrawal of the stresses, enzyme activity decreased to the control level. The enhanced SPX activity suggests intensification of cell wall component synthesis, increasing cell wall rigidity.

DISCUSSION

Cucumber is a crop plant relatively susceptible to unfavorable environmental conditions; it is often chosen for studies investigating the reaction to one or more stress factors (Yinan et al., 2005; Kataria et al., 2007).

Our earlier work estimated ROS level, antioxidant system activity and polyamine level in water-stressed cucumber seedlings (Kubiś, 2008). Under natural conditions stress factors hardly ever act separately. Water deficit stress is often accompanied by excess radiation (light and UV-B) and heat, so the reaction of cucumber plants to a combination of soil drought and enhanced UV-B radiation is a subject of interest.

Progressive stress conditions caused a slow decrease of leaf water content (Fig. 1a) and an increase of dry weight (Fig. 1b) in cucumber leaves, the effect being most pronounced under drought treatment. Water deficit, UV-B radiation and co-stresses induced changes in scavenging system enzymes (Fig. 2). SOD (Fig. 2a), GPX (Fig. 2b), and GR activity (Fig. 2c) generally increased, but the stress-dependent alterations did not occur at the same time of stress treatment: SOD activity increased earlier (day 2) than GPX and GR (days 5 and 7).

These results support the suggestion (Elstner, 1991) that SODs constitute the first line of defense against oxidative stress. After 4 days of water stress treatment (soil drought), a similar effect, increased SOD activity, was recorded in barley (Smirnoff,

1993; Acar et al., 2001), tomato (Bowler et al., 1992), sorghum (Jagtap and Bhargava, 1995) and wheat (Sairam and Srivastava, 2001). Short-term experiments (up to 3 days) using PEG as an osmoticum produced no clear results on the effect of water deficit on SOD activity (Ahuja and Kaur, 1985; Badiani et al., 1990; Quartacci and Navari-Izzo, 1992), while rapid and intensive desiccation of seedlings subjected to 24 h water stress triggered down-regulation of SOD activity in earlier studies using barley (Kubiś, 2005) and cucumber (Kubiś, 2008). These results mean that treatment time is significant in the triggering of SOD activation.

Earlier work on barley found similar increases of GPX and GR activity (Kubiś, 2001, 2003) and cucumber (Kubiś, 2008). Bandurska (2002) reported that two barley genotypes subjected to osmotic stress showed similar changes in guaiacol peroxidase activity. Some authors measured guaiacol peroxidase activity and found neither an increase nor a decrease during water deficit (Smirnoff, 1993).

In this study, antioxidant activity was generally altered in UV-B-treated plants, but the effect was weaker than in drought-treated ones. Elevated SOD, GPX and GR under excess UV-B radiation were noted in cucumber cotyledons (Kataria et al., 2007), winter wheat seedlings (Yang et al., 2007), mung bean cultivars (Agraval and Rathore, 2007) and spinach chloroplasts (Lei et al., 2008). The oxidative stress conditions caused by different treatments vary; they all seem to be related to overproduction of reactive oxygen species, but they engage different pathways of the antioxidant system for their removal (Kubo et al., 1999).

The combination of two stresses, and drought, elevated the activity of the investigated antioxidant enzymes. The ratios of enzyme activation were significantly lower than under water deficit or under UV-B radiation separately, especially on later days of co-treatment. These results suggest that these co-stresses functioned synergistically, with one of them reducing the changes caused by simultaneous application of the other stress. Very few data are available on the effects and interrelationships between drought and ultraviolet-B radiation. Alexieva et al. (2001) observed similar effects of co-stresses in pea and wheat seedlings. In contrast, other parameters were reduced by a combination of two stresses in comparison with single stresses in spring wheat: plant growth, photosynthetic capacity, pigment content, biomass and yield (Feng et al., 2007). We also noted a decrease in leaf biomass (data not shown), but an increase in dry weight, especially under UV-B as compared with the other treatments, and this could be related to increased cell wall rigidity. The enhanced SPX activity suggests intensification of cell wall component synthesis and a consequent increase in cell wall rigidity, which can contribute to drought tolerance (Clifford et al., 1998; Garciá at al., 2000). The values of the physiological parameters we measured indicate that the two environmental stresses acted synergistically to trigger protective mechanisms, activating the antioxidant system, but that the application of either stress reduced the effect of application of the other stress.

We suggest that in plants grown in the natural environment, where usually more than one stress factor prevails, the same effect should be expected: the stressors will act synergistically.

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