Effect of UV-B Radiation on Antioxidative Enzyme Activity in Cucumber Cotyledons

Magdalena Rybus-Zając[*] and Jan Kubiś

Department of Plant Physiology, University of Life Sciences in Poznań,
Wołyńska 35, 60-637 Poznań, Poland

Ultraviolet-B (UV-B) radiation negatively affects plant cells, causing reactive oxygen species (ROS) to be generated. To study the effects of increased UV-B exposure on antioxidant processes, we exposed germination-stage cucumber seedlings to increased ultraviolet radiation and analyzed hydrogen peroxide content and the activity of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX). Enzymatic antioxidant system activity generally increased after UV-B supplementation. Activation of CAT, GPX and SPX in cucumber cotyledons correlated positively with increases in SOD activity and hydrogen peroxide content. The results indicate that ROS accumulated despite higher engagement of the enzymatic antioxidant system, and that elevated UV-B radiation triggered oxidative stress in the cucumber cotyledons.

Key words: Catalase, guaiacol peroxidase, H_2O_2, superoxide dismutase, syringaldazine peroxidase.

INTRODUCTION

Ozone depletion in the stratosphere, caused by trace gases such as chlorofluorocarbons (CFCs) and NO_x, results in increased levels of ultraviolet-B radiation (UV-B, 280-315 nm) reaching the Earth's surface. Stratospheric ozone recovery was not expected before 2010, and will be slowed due to the effect of greenhouse gas emissions (Kakani et al., 2003). The decreased ozone levels are expected to recover to 1970 levels by 2050 (UNEP 2002). Current levels of UV-B during the cropping season are anywhere between 2 and 12 kJ m^{-2} per day on the Earth's surface, an increase of 6–14% UV-B radiation over pre-1980 levels, and the effects of enhanced radiation may increase in future years (Madronich et al., 1998, UNEP 2002). This range of radiation has a multifaceted, most often negative, effect on crops. Plants must adapt to the deleterious effects of UV-B radiation because they depend on sunlight for photosynthesis and therefore cannot avoid exposure to UV-B radiation. The intensity of damage caused by stress depends on the plant species and its development phase, anatomical and morphological characteristics, environmental conditions, as well as the dose and duration of UV-B. In addition to directly damaging DNA, UV-B radiation frequently triggers oxidative stress through the formation of reactive oxygen species (ROS), which in turn cause enhanced lipid and protein oxidation (Yanarelli et al., 2006a). Thus, oxygen toxicity is observed to increase in plant cells (Langebartels et al., 2002, Mittler 2002; Jain et al., 2004), accompanied by the formation of elevated amounts of ROS (Lamb and Dixon, 1997; Foyer and Noctor, 2003, 2005), which include superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) (Scandalios 2002). The formation of H_2O_2 occurs in such physiological processes as photosynthesis and respiration. This metabolite participates indirectly in generation of structural barriers or serves signaling functions (Mittler, 2002; Neil et al., 1999, 2002; Veljavic-Jovanovic et al., 2002). The specific signaling pathway that controls the expression of the set of genes engaged in defense against UV-B is rapidly up-regulated (Agrawal et al., 2009). H_2O_2 is rather stable, but other ROS are relatively short-lived in cells (Asada, 1999). In response to ROS generation the antioxidant system is triggered; it includes superoxide dismutase (SOD), catalase (CAT) and peroxidases (POXs), as well as low-molecular antioxidants (Scandalios 2002). Superoxide dismutase generates

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e-mail: magrybus@jay.up.poznan.pl

H_2O_2, and catalase is one of the main enzymes that scavenge hydrogen peroxide. Peroxidases, present in almost every organism, catalyze oxidation of a wide range of substrates, mainly phenols, dependent on hydrogen peroxide decomposition. In plants they serve numerous physiological functions including hydrogen peroxide detoxification, lignin biosynthesis, hormonal signaling and stress response. Almost every organism contains POXs (Passardi et al., 2004).

Cucumber is a plant highly sensitive to stress factors. It is also one of the more common vegetable species. When grown in the field its cotyledons are at real risk of exposure at early stages of germination to stresses such as elevated UV radiation. We analyzed hydrogen peroxide levels and the activity of superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), syringaldazine peroxidase (SPX) in cucumber cotyledons under exposure to elevated ultraviolet-B radiation. We assessed the sensitivity of the species at germination stage.

MATERIAL AND METHODS

The experimental material consisted of seedlings of cucumber cv. Dar grown on perlite under controlled growth conditions: 25°C, 14 h photoperiod, and 120 μmol m^{-2} s^{-1} photon flux density (400–700 nm). Seven-day-old seedlings were subjected to UV-B irradiation supplied by Philips TL 20 W/01 RS lamps, max. 315 nm, 16 kJ m^{-2} day^{-1} intensity, for 8 h per day (3.25 μmol m^{-2} s^{-1} photon flux density) for 3–10 days.

Hydrogen peroxide concentration was assayed using the method of Messner and Boll (1994). Cotyledon samples were ground in 3 ml cold K phosphate buffer (pH 7.0) containing 0.02 g Polyclar AT. The homogenate was centrifuged for 25 min at 15,000 g at 4°C. The reaction mixture contained 1.5 ml extract, 0.15 ml K phosphate buffer (pH 7.0), 50 ml horseradish peroxidase (1 mg/1ml 100 mM K phosphate buffer, 60 units/mg) and 0.05 ml 0.05 M ABTS (2,2'-Azino-bis(3-ethylobenzothiazoline-6-sulfonic acid) diammonium salt). Absorbance increase according to the H_2O_2 level at 415 nm was measured after 3 min and compared to the standard curve of freshly prepared 0–30 nM H_2O_2 solutions in 100 mM K-phosphate buffer (pH 7.0). The results are given in nmol hydrogen peroxide (H_2O_2) per mg protein.

Superoxide dismutase activity was assayed using the method of Beauchamp and Fridovich (1971), which measures inhibition in photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. Cotyledons were ground in 3 ml cold solution containing 0.05 M Na phosphate buffer (pH 7.0), 1% PVP (polyvinylpolypyrrolidone), 1 mM EDTA (ethylenediaminetetraacetic acid) and 0.5 M NaCl, and centrifuged for 25 min at 15,000 g at 4°C. The supernatant was used as enzyme extract. The 3 ml reaction mixture contained 0.05 M Na phosphate buffer (pH 7.8), 0.1 mM EDTA, 97 mM L-methionine, 120 mM riboflavin, 2 mM NBT and 30 μl enzyme extract. The reaction was carried out for 10 min under a fluorescent lamp. One unit of activity was estimated as the quantity of enzyme reducing absorbance to 50% of that of tubes lacking the enzyme. Total enzyme activity was expressed in units per mg protein.

For catalase and peroxidase activity the cotyledons were ground in 0.1 M phosphate buffer (pH 7.0) containing 0.5% polyethylene glycol (PEG 6.000) and 40 mg Polyclar AT, and centrifuged at 10,000 g for 15 min at 4°C, and the supernatant was used as enzyme extract.

Catalase activity was determined by measuring H_2O_2 consumption (Dhindsa et al., 1981) in reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 15 mM H_2O_2 and extract, at 240 nm against a calibration curve. Activity was expressed as μM decomposed H_2O_2 per mg protein.

Guaiacol peroxidase was determined according to the method of Hammerschmit et al., (1982). The reaction mixture contained 0.2 mM guaiacol, 0.09 mM H_2O_2 and enzyme extract. H_2O_2-dependent oxidation of guaiacol is followed by an increase of absorbance at 470 nm. Enzyme activity was calculated as the increase of absorbance (ΔA) per mg protein.

Syringaldazine peroxidase was determined according to Imberty et al. (1985). The reaction mixture contained 0.1 M phosphate buffer (pH 6.0), 0.5 mM H_2O_2, syringaldazine (3.1 mg dissolved in 1 ml methanol and mixed 1:2 with dioxane) and enzyme extract. Enzyme activity was calculated as the absorbance increase at 530 nm (ΔA) per mg protein.

Protein content was determined by Bradford's (1976) method.

Photosynthesis rate was analyzed for whole cucumber seedlings by measuring CO_2 loss in a closed system using an AirTECH 2500-P CO_2 analyzer. The results were expressed as mg CO_2 consumed per g fresh weight.

Statistical analysis. The experimental data were subjected to one-way ANOVA, and significant differences between means were determined by Tukey's multiple range test ($p < 0.05$, $p < 0.01$).

RESULTS

Elevated UV-B radiation applied at the germination stage led to a time-dependent increase of hydrogen peroxide level in cucumber cotyledons, by 93%, 70% and 73% respectively at 5, 7 and 10 days of stress versus the controls (Fig. 1). Thus the
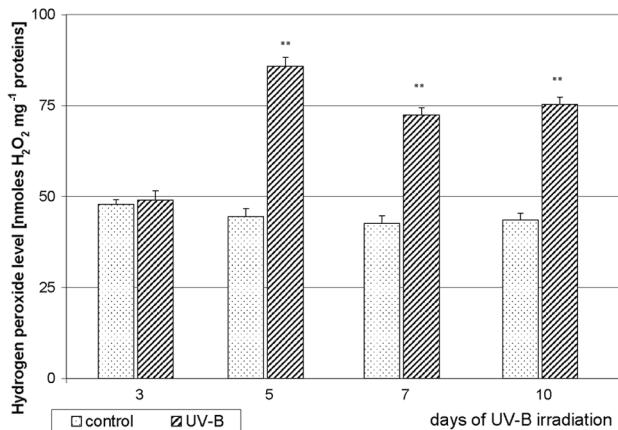
Fig. 1. Effect of progressive UV-B on hydrogen peroxide level in cucumber cotyledons. Significant difference between stressed plants and control: $^*p<0.05$, $^{**}p<0.01$
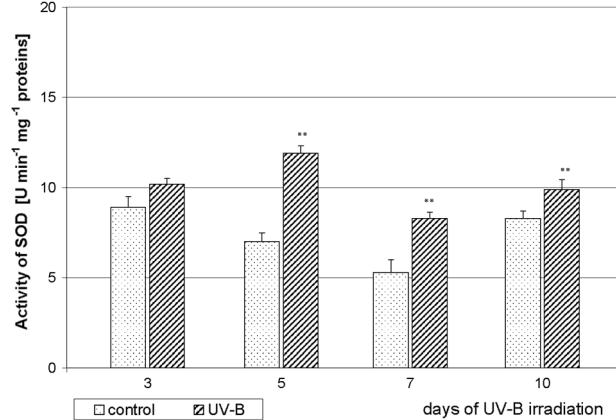

Fig. 2. Effect of progressive UV-B on superoxide dismutase (SOD) activity in cucumber cotyledons. Significant difference between stressed plants and control: $^*p<0.05$, $^{**}p<0.01$
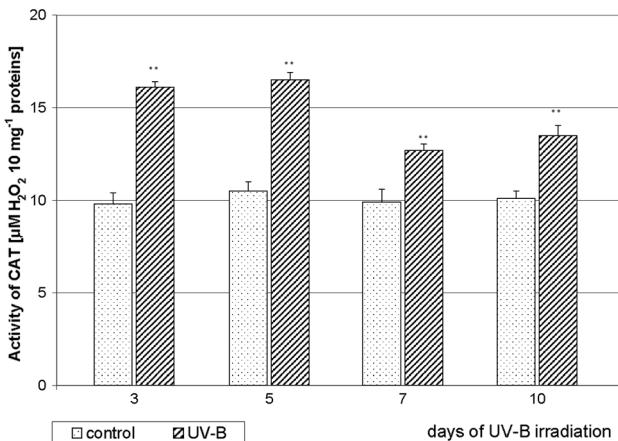

Fig. 3. Effect of progressive UV-B on catalase (CAT) activity in cucumber cotyledons. Significant difference between stressed plants and control: $^*p<0.05$, $^{**}p<0.01$
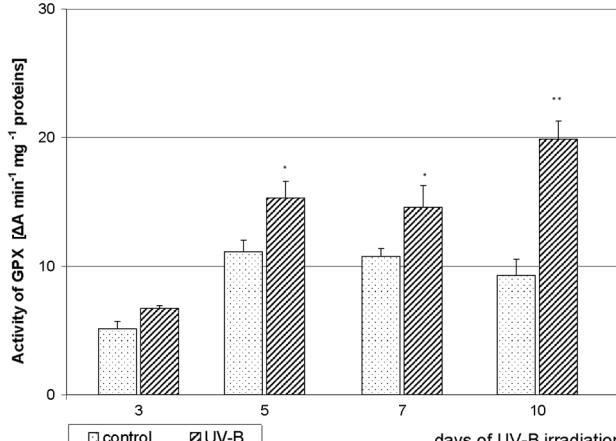

Fig. 4. Effect of progressive UV-B on guaiacol peroxidase (GPX) activity in cucumber cotyledons. Significant difference between stressed plants and control: $^*p<0.05$, $^{**}p<0.01$

changes were already considerable at day 5 of stress. Superoxide dismutase activity also increased, by 14% (day 3 of stress), 70% (day 5), 56% (day 7) and 19% (day 10) versus the controls (Fig. 2). Except for the last day of the stress these changes were correlated with higher levels of hydrogen peroxide. In control plants the hydrogen peroxide level remained stable.

UV-B irradiation stress induced increased catalase activity in cucumber cotyledons as compared with the stable levels in the controls (Fig. 3). Under UV-B exposure it reached 16.1 units (day 3, vs 9.8 units in control), 16.5 units (day 5, vs 10.5), 12.7 units (day 7, vs 9.9) and 13.5 units (day 10, vs 10.1).

Guaiacol peroxidase activity at successive days of elevated UV-B irradiation amounted to 6.73 units (day 3, vs 5.12 units in control), 15.31 units (day 5, vs 11.13), 14.58 units (day 7, vs 10.75) and 19.90 units (day 10, vs 9.28) (Fig. 4). Those levels in UV-B treated plants are 31%, 37%, 36% and 114% higher than the values from the respective controls.

Syringaldazine peroxidase activity in seedlings exposed to UV-B irradiation reached 18.62 units (day 3, vs 19.90 units in control), 32.56 units (day 5, vs 21.56), 36.62 units (day 7, vs 22.00), and 53.55 units (day 10, vs 38.78) (Fig. 5). Those levels in UV-B-treated plants are 51% (day 5), 66.5% (day 7) and 38% (day 10) higher than in the controls.

Under increased UV-B irradiation the photosynthesis rate decreased in a time-dependent manner versus the stable levels in the controls (Fig. 6); in UV-B treated cucumber seedlings, CO_2 assimilation
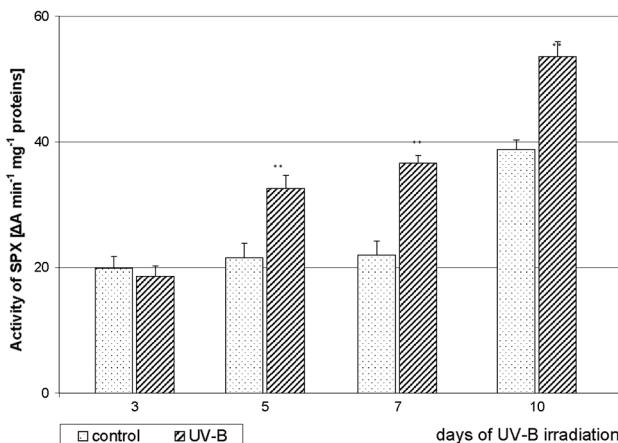
Fig. 5. Effect of progressive UV-B on syringaldazine peroxidase (SPX) activity in cucumber cotyledons. Significant difference between stressed plants and control: $p<0.05$, $p<0.01$

Fig. 6. Effect of progressive UV-B on photosynthesis rate in cucumber cotyledons. Significant difference between stressed plants and control: $p<0.05$, $p<0.01$

declined to 2.85 units (day 3, vs 3.08), 2.18 units (day 5, vs 2.85), 2.09 units (day 7, vs 3.02) and 2.82 units (day 10, vs 2.89).

DISCUSSION

In plants the most frequent effect of exposure to elevated UV radiation is oxidative stress and ROS overproduction, in which a significant role is played by hydrogen peroxide. H_2O_2 is an important signalling molecule (Foyer and Noctor, 2003, 2005) which induces further responses of plants to environmental stresses, both biotic and abiotic (Dat et al., 2000; van Breusegem et al., 2001; Neil et al., 2002; Bolkhina et al., 2003). It is generated via superoxide, presumably in a non-controlled manner, during electron transport processes such as photosynthesis and mitochondrial respiration (Alscher et al., 2002; Neill et al., 2002). Potential sources of hydrogen peroxide synthesis include NADPH oxidase, amine oxidase, oxalate oxidase and flavin-containing oxidases (Bolwell and Wojtaszek, 1997; Bolwell at al., 2002).

We noted enhanced hydrogen peroxide content after enhanced UV-B irradiation, with correlated increases of SOD activity. This suggests that under UV-B stress H_2O_2 was formed intensively as a result of enzymatic superoxide anion dismutation. Similar changes have been reported by Baumbusch et al. (1998), Dai et al. (1997), Kubo et al. (1999), Rybus-Zając (2005), and Kubiś and Rybus-Zając (2008), but those investigations used plants during intensive vegetative growth. Rarely analyzed is the plant response at the seedling stage, especially at a very early stage which is connected with high oxidative activity and the involvement of cotyledons (Jain et al., 2004). There are reports on changes in SOD activity
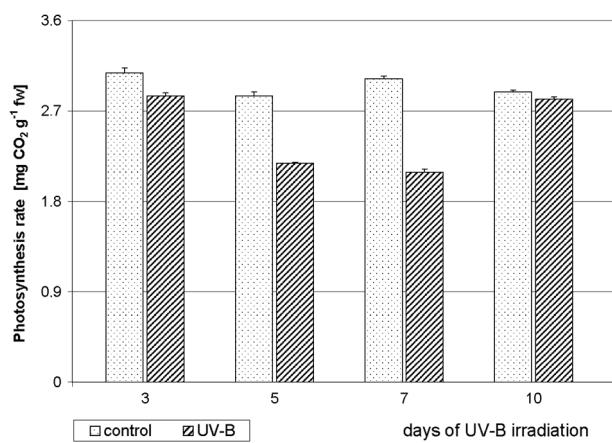
and overproduction of H_2O_2 as a result of exposure to UV-B radiation in sunflower cotyledons (Yanarelli et al., 2006b) and *Picea asperata* seedlings (Han et al., 2009). In cucumber cotyledons irradiated with ultraviolet UV-C (254 nm) or UV-A+UV-B (280-360 nm), Watanabe at al. (2006) detected H_2O_2 accumulation and suggested that UV irradiation causes oxidative damage to DNA in plant cells.

Oxidative stress arises from an imbalance in the generation and decomposition of ROS, with more ROS (such as H_2O_2) being produced than are metabolized (Neill et al., 2002). Recent work has confirmed that hydrogen peroxide is a signaling molecule which mediates responses to abiotic stresses in plants; enhanced UV-B increased the efficiency of the antioxidant defense system, including UV-B-absorbing compounds and antioxidant enzyme activity (Han et al., 2009). The H_2O_2 accumulation we observed in cucumber cotyledons was accompanied by an increase in the activity of antioxidant system enzymes catalyzing reactions of hydrogen peroxide removal: CAT, GPX and SPX. Balakumar et al. (1997) reported increased catalase activity in tomato plants after UV-B irradiation. Increased peroxidase activity under the influence of UV-B irradiation has been reported frequently in, for example, barley (Mazza et al., 1999), sugar beet (Panagopoulas et al., 1990), sunflower cotyledons (Yanarelli at al., 2006b), long green cucumber cultivar (Kataria et al., 2007), wheat and mung bean (Agrawal and Ratore, 2007), spinach (Lei at al., 2008), *Picea asperata* seedlings (Han et al., 2009) and peanut (Tang et al., 2010). Sometimes, however, the activity of this enzyme is found to decline when the irradiation dose is too high (Rao et al., 1996). UV-B radiation induces increased activity of other antioxidant system enzymes of cucumber cotyledons as well; Kataria et

al. (2007) reported increased glutathione reductase and ascorbic acid peroxidase activity.

UV-B stress influenced antioxidant activity in the cell wall region in cucumber cotyledons, as seen in increased syringaldazine peroxidase levels. Intensification of cell wall component synthesis can increase cell wall rigidity, which in turn can boost tolerance to drought, UV-B and co-stresses (Clifford et al., 1998; Garciá at al., 2000; Kubiś and Rybus-Zając, 2008).

Enhanced UV-B reduced photosynthesis in our tested cucumber cotyledons. Previous work also showed negative effects of UV-B on photosynthetic processes, resulting in decreased biomass production (Correira et al., 2005; Agrawal and Rathore, 2007; Han et al., 2009).

The disturbances recorded in seedlings under elevated UV-B radiation indicate that accumulation of the signaling molecule hydrogen peroxide triggered further defense mechanisms, altering the antioxidant defense capacity and thus minimizing the negative impact of UV-B on the plants. The higher concentration of H_2O_2 induced oxidative stress and boosted the antioxidant defense system.

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