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Effects of Salicylic Acid on Oxidative Stress and UV-B Tolerance in Cucumber Leaves

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The effect of salicylic acid (SA) on antioxidant system and protective mechanisms against UV-B induced oxidative stress was investigated in cucumber (*Cucumis sativus* L.) leaves. UV-B radiation and SA were applied separately or in combination to first leaves of cucumber seedlings, and dry matter accumulation, lipid peroxidation and activities of antioxidant enzymes were measured in both dose and time-dependant manner. UV-B exposure showed reduced levels of fresh weight and dry matter production, whereas SA treatment significantly increased them. SA noticeably recovered the UV-B induced inhibition of biomass production. UV-B stress also affected lipid peroxidation and antioxidant enzyme defense system. Malondialdehyde(MDA), a product of lipid peroxidation, was greatly increased under UV-B stress, showing a significant enhancement of a secondary metabolites, which may have antioxidative properties in cucumber leaves exposed to UV-B radiation. Combined application of UV-B and SA caused a moderate increase in lipid peroxidation. These results suggest that SA may mediate protection against oxidative stress. UV-B exposure significantly increased SOD, APX, and GR activity compared with untreated control plants. Those plants treated with 1.0 mM SA showed a similar pattern of changes in activities of antioxidant enzymes. SA-mediated induction of antioxidant enzyme activity may involve a protective accumulation of H_2O_2 against UV-B stress. Moreover, their activities were stimulated with a greater increase by UV-B + SA treatment. The UV-B + SA plants always presented higher values than UV-B and SA plants, considering the adverse effects of UV-B on the antioxidant cell system. ABA and JA, second messengers in signaling in response to stresses, showed similar mode of action in UV-B stress, supporting that they may be important in acquired stress tolerance. Based on these results, it can be suggested that SA may participates in the induction of protective mechanisms involved in tolerance to UV-B induced oxidative stress.

Key Words : Antioxidative enzyme, Cucumber (*Cucumis sativus* L.), Lipid peroxidation, Oxidative stress, Salicylic acid, UV-B

1. Introduction

Since ultraviolet-B (UV-B ; 280-320 nm) is the wavelength range affected by changes in stratospheric ozone depletion, attention has been focused on the harmful effects of UV-B radiation on living organisms. Because UV-B radiation is one of the major abiotic stresses limiting plant yield and distribution in many regions of the world, it has been the focus of much research. Both the magnitude and extent of the response of plants to UV-B radiation are influenced by a number of other factors, including physiological and developmental status of the plant, as well as the amount and spectral composition of UV-A and visible radiation[1].

UV-B radiation induces a multitude a rapid and longterm morphogenic responses which are mediated by an unknown number of unidentified photosensory systems[2]. Some UV-B driven morphogenetic responses may not involve a dedicated photosensory system, but rather are a consequence of UV-B induced change in secondary metabolism. UV-B is absorbed effectively by nucleic acid and other sensitive targets, potentially causing harmful photochemical effects. Numerous studies have demonstrated several detrimental effects

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of UV-B on plants, including DNA damage, inhibition of photosynthetic activity and growth[3,4]. Therefore, increased UV-B is expected to have a negative impact on UV-B sensitive terrestrial plants. In order to prevent these harmful effects of UV-B radiation, plant have developed several defense mechanisms. Laboratory and field experiments have shown that different mechanisms are involved in the protection of plant tissues against UV-B radiation damage. Protection against UV-B radiation may be afforded by substances, mainly flavonoids and related phenolic compounds, which have the capacity to not only shield the tissue by UV absorption, but also to scavenge the reactive oxygen species generated[5]. Plants accumulate UV-absorbing phenolic compounds, mainly in the vacuoles of epidermal cells, in order to prevent the penetration of UV-B into the internal tissue[6]. Leaf UV-B absorbing potential varies considerably among different plant species and life forms, possibly indicating a different resistance against environmental stress factor.

Salicylic acid (SA), a natural plant phenolic compound, has been implicated in signaling in response to several stresses. In recent years SA has been included in protection against chilling, UV-B and ozone in addition to its well documented involvement in defense against pathogen[7]. Several studies have also found that SA plays a major role in modulating plant responses to various abiotic stresses[8,9]. SA has been suggested as functioning in the induction of antioxidant defense response and maintenance of the glutathione pool, both possible indications of its involvement in plant protection against oxidative stress[10]. The UV-B induction of antioxidative enzymes is assumed to constitute a defense response of plants to free radicals generated by UV-B. In addition, it has recently been suggested that phenolic compounds contribute the the UV-B adaptation of plants not only by UV-absorption but also through their other functions, such as radical scavenging[11]. There is also evidence for interactions between SA and antioxidant system. The increasing evidence include that SA may be a signal that interact with active oxygen species such as H_2O_2 during biotic and abiotic stresses, and SA can directly inhibit the activity of the H_2O_2-scavenging enzymes[12]. Further investigation of these newly discovered effects of SA may prove rewarding, as plants are believed to use the antioxidant enzyme to defend against oxidative stress and enhance their abiotic stress tolerance in plants. However, the relevant literature is still little available and involves diverse plant systems and methods of SA application.

In the present paper we provide further evidence of the effects of SA on UV-B tolerance and protection against UV-induced oxidative stress. We compared changes in the antioxidant system using cucumber seedling system during UV-B radiation induced by either SA treatment or UV-B radiation to explore whether SA can reduce or ameliolate the adverse effects of UV-B radiation.

2. Materials and Methods

2.1. Plant material and growth conditions

Seeds of cucumber (*Cucumis satius* L,) were sterilized with 10% NaOCl for 10 min and rinsed thoroughly with sterile distilled water. The seeds were then sown in a mixture of vermiculite, peat moss and perlite in plastic pots (7×11cm), and were watered with a 1/2-strength Hoagland solution. The seedlings were reared in a growth chamber at a 25 ± 1℃ with 70% relative humidity and a 12 h photoperiod provided by 160 μmol m^{-2} s^{-1} PAR. The plants were watered every 2 days. At 15 days after sowing, seedlings were transferred to another chamber to be grown under a 20/15℃ (light/dark) temperature regimes.

Over the course of the growing period, the plants were exposed to UV-B and SA alone or in combination for 10 days. SA was applied to the 15-d-old plants during the UV-B treatment. Stock of SA(Sigma Co.) had been prepared in a small volume of ethanol, then diluted to the three treatment concentrations (0.1, 0.5 and 1.0 mM) in a 2 mM sodium phosphate buffer (pH 7.0). The buffer solution alone was applied on control plants.

2.2. Ultraviolet-B radiation treatments

15-d-old seedlings were used for the UV-B treatments. Control plants were grown under visible light only, while treated plants were irradiated with supplementary UV-B during the 12 h light periods. UV-B was provided by two fluorescent UV-lamps (VL-6, Viber lourmat France sun lamp), suspended 53 cm above the plant seedlings. The UV-B fluence rate, at the height of seedlings, was measured to be 6W

m^{-2} s^{-1} using a UV spectroradiometer (Li-1800, Lycosa). For time-course analysis, the seedlings were harvested at different time intervals for the estimation of MDA and antioxidant enzyme activity.

2.3. Growth analysis

Fresh weight and dry weight of the cucumber primary leaves were determined at daily intervals starting on the 1 day after planting and continuing through the 7 days. Leaf fresh weight was determined by means of a electrobalance (Model H 51, Saritorius GmbH, Germany), and leaf dry weight was obtained by drying sample for 72 h at 80℃ in a drying oven and reweighing.

2.4. Lipid peroxidation determination

Lipid peroxidation was quantified by measuring the amount of malondialdehyde (MDA), a product of unsaturated fatty acid peroxidation. MDA concentration was estimated by the method of Zhao et al.[13]. Fresh leaves(0.5g) were homogenized in 10 ml 5% (w/v) trichloroacetic acid. Homogenates were then centifuged at 4,000 g for 10 min. The supernatant (2 ml) was mixed with 1 ml of 0.67% (w/v) thiobarbituric acid, and then boiled at 100℃ for 20 min and cooled immediately in an ice bath. After centrifugation at 12,000 g for 10 min, the absorbance of the superntant at 532 and 620 nm was determined using a spectrophotometer. The MDA content was calculated by using the extinction coefficient at 155 mM^{-1} cm^{-1}. MDA level is routinely used as an index of lipid peroxidation and was expressed as μmol g^{-1} FW.

2.5. Assays of antioxidant enzymes

Primary leaves exhibiting no visible injury symptoms were harvested and used to determine the activities of SOD, APX and GR. Leaves were homogenized in 10 mM of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ascorbate and 0.5 mM EDTA in pre-cooled mortar and pestle and centrifuged at 22,000 g for 30 min at 4℃. The supernatant was used as a crude extract for enzyme assays.

Superoxide dismutase (SOD) activity was assayed as described by Beyer and Fridovich[14]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 μM nitroblue tetrazolium (NBT), 0.9 μM riboflavin, 0.025% (w/v) Triton X-100, and the appropriate amount of leaf extract. The A_{560} was recorded after a 7-min illumination period. One unit of SOD is defined as the amount of enzyme required to inhibit the photoreduction of NBT by 50%. Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada[15], in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and crude extract. The decrease in absorbance was read at 290 nm. The assay for glutathione reductase (GR) activity was performed as described by Tanaka et al.[16], using a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 0.2 mM NADPH, 0.5 mM GSSG, and the leaf extract. GR activity was determined by following the oxidation of NADPH at 340 nm.

2.6. Statistical analysis

The experiments were performed three times and the mean of the three experiments and standard error are shown. Significance of differences between treatments was statistically evaluated by SD and Student's *t*-test methods.

3. Results and Discussion

3.1. Effects of SA on UV-B induced oxidative damage

To examine whether SA treatment could protect cucumber seedlings from UV-B induced oxidative damage, the plants were treated without or with SA (0.1, 0.5 and 1.0 mM) under UV-B exposure. In response to UV-B radiation, cucumber seedlings showed signs of UV-B damage and necrosis, although they still had growing, green shoots. The incidence of leaf injuries, e.g. fresh weight and dry weight were reduced in plants exposed to UV-B alone, while those that had been treated with SA were less affected (Table 1). The leaf biomass increased with the increasing SA treatment. Treatment with SA at 1.0 mM SA increased the leaf fresh weight and dry weight by 13% and 11%, respectively. Decreased biomass in response to supplemental UV-B have been reported and leaf biomass was also lowest in ambient UV-B, although not significantly. UV-B radiation is generally detrimental to plants, e.g. reducing growth and biomass, and can also cause changes in biomass allocation[17]. We speculate that reduced biomass production seems

Table 1. Effect of salicylic acid on UV-B induced fresh weight and dry weight of cucumber primary leaves

Treatment	Fresh weight (mg)		Dry weight (mg)	
	Control	UV-B	Control	UV-B
Control	242.4±0.24	229.6±0.34	21.0±0.51	19.9±0.19
0.1 mM SA	218.6±0.54	244.8±0.21	18.8±0.35	21.0±0.22
0.5 mM SA	258.6±0.34	285.6±0.57	20.9±0.33	25.1±0.34
1.0 mM SA	269.9±0.55	290.4±0.11	20.4±0.21	25.5±0.21

to be due to a adverse effect of UV-B radiation on the photosynthetic process, and may potentially be explained by derived chl a fluorescence parameters. Furthermore, plants exposed to high levels of PAR and UV-B have been reported to be especially susceptible to formation of active oxygen species which are potential photodamaging agents[18].

Both SA and UV-B + SA significantly increased levels of fresh weight and dry weight, indicating that reduced biomass production under UV-B stress was ameliolated by SA. Concentration as high as 3.5 mM/L SA were necessary to reduce growth and fresh weight of *Vicia faba* seedlings[19]. In contrast, SA enhanced elongation growth of tree seedlings and soybean shoots and roots[20].

Exposure of seedlings to excess UV-B led to lipid peroxidation in leaves. UV-B significantly increased MDA concentration which represents the state of membrane lipid peroxidation in the first leaves, confirming an oxidative stress (Fig. 1). MDA level was enhanced from 48 h after the start of UV-B treatment, attaining 3.5-fold increases compared with the levels of controls after 7 d of irradiation. A significant rise in MDA level was observed at 96 h with UV-B

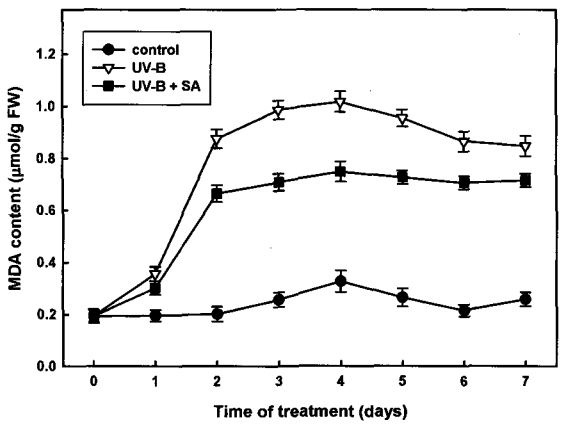

Fig. 1. Changes in MDA content in cucumber primary leaves treated UV-B and SA alone or in combination.

radiation. This result agrees with a previous study where tomato seedlings were exposed to UV-B in the field[21]. In contrast, enhanced UV-B radiation did not affect lipid peroxidation[22]. An increase in lipid peroxidation by UV-B exposure indicates antioxidant role of secondary metabolites in cucumber leaves exposed to UV-B radiation.

Similarly, a rapid increase in MDA level was observed in the UV-B + SA treatment. Moderate increase in MDA levels by SA treatment can cause a large induction of antioxidant system, thereby leading to enhanced UV-B tolerance in cucumber plants. These results suggest that SA may implicate in protection from UV-B induced oxidative damage.

3.2. Effects of SA on the antioxidant system

In order to examine the effect of UV-B stress on the activities of several antioxidative enzymes in cucumber first leaves, time-dependent variations in the activities of antioxidative enzymes (SOD, APX and GR) were measured over a period of 7 d in the UV-B treated leaves. Activities of SOD, APX and GR were markedly increased in response to 7 d of exposure to UV-B alone, compared with untreated control plants. In the control seedlings slight fluctuation in the SOD activity was observed (Fig. 2), while UV-B treatment resulted in significant effect on SOD activity and the response to stimulation lasted for 7 d. Exposure of seedlings to UV-B began to enhance the activity of SOD drastically from 48 h after the start of UV-B treatment, attaing 4.5-fold increases compared with controls after 7 d of irradiation. The APX activities in leaves were low during the initial 24 h after exposure of seedlings to UV-B radiation followed by a significant increase by 2.1-fold after 7 d of induction (Fig. 3). In contrast, the activities of GR showed a significant decline during the initial 48 h after exposure of seedlings to UV-B radiation followed by a gradual increase lasted to 7 d with small variation (Fig. 4).

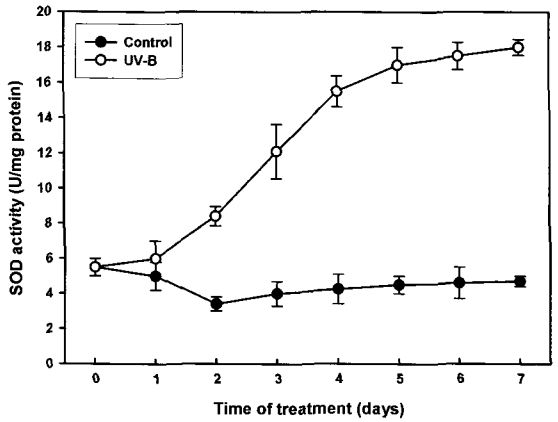

Fig. 2. Changes in SOD activity in cucumber primary leaves exposed to UV-B radiation.

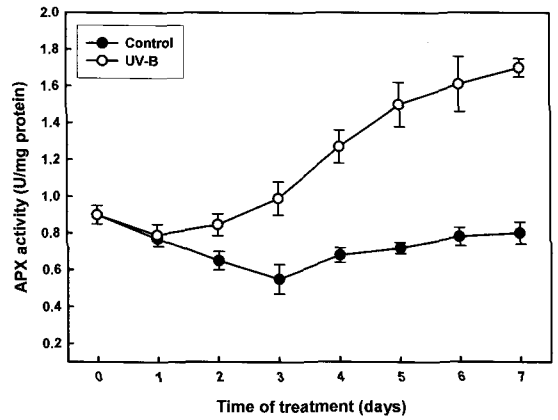

Fig. 3. Changes in APX activity in cucumber primary leaves exposed to UV-B radiation.

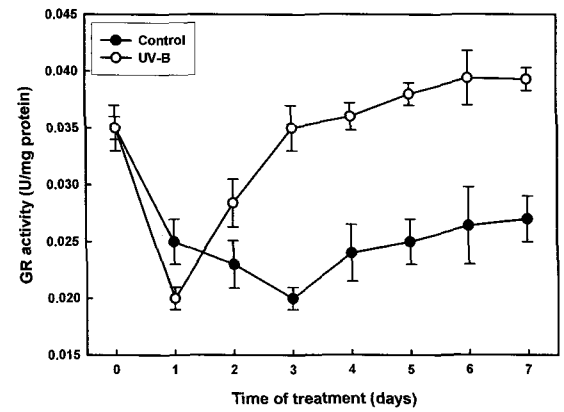

Fig. 4. Changes in GR activity in cucumber primary leaves exposed to UV-B radiation.

In the present experiments using cucumber first leaves, activities of SOD and APX were significantly enhanced by UV-B radiation, while the activity of GR was not significantly affected by UV-B treatment. Although the enzymes were damaged in seedlings in their first developmental stage, SOD activity was restored and reached maximal levels at 5 d. APX activities were progressively increased by UV-B exposure, suggesting that the antioxidative capacity stimulated by UV-B could be the involved in conversion of H_2O_2 and O_2. The low levels of APX activity during the initial period of UV-B treatment suggest that plants require a time lag for induction of the enzyme.

To examine the possible involvement of SA in protection against UV-B induced oxidative stress, leaf excised from UV-B irradiated or non-irradiated cucumber primary leaves was treated with various concentrations of SA. Treatment with SA at 0.1-1.0 mM progressively increased the activities of antioxidative enzymes, showing that the response of stimulation lasted for 7 d and the enzyme activities exceeded the controls (Fig. 5). SA caused increases in SOD, APX and GR activities, being 247%, 85% and 114% higher than controls in plants grown on 1.0 mM SA, respectively. Increases in GR activity following SA treatment have also been reported for pea seedlings[23] and hydroponic maize[24], while no effects of SA on APX activity were found in maize[24].

There was significant increase in antioxidant enzyme activity at combined stress (UV-B + SA), ensuring that SA may lead to enhanced UV-B tolerance. The application of SA to UV-B irradiated plants increased the enzymatic activities that resulted from UV-B stress, with the effect being more pronounced in the 1.0 mM treatment (Fig. 6). The SOD activity increased in the presence of UV-B and this increase was more accentuated in UV-B + SA treatment, resulting in a 37.4% increase compared with level of SA alone for 1.0 mM SA. APX and GR activities were also stimulated with 19.7% and 7.2% increase compared with level of SA alone by UV-B + SA treatment, respectively. SA caused significant increase in antioxidant enzyme activity of three to four times for 1.0 mM SA, while these enzyme activity was not affected by 0.1 mM SA. The data demonstrate that the oxidative damage initially seen under UV-B radiation can be rapidly repaired by SA treatment. This suggests that the molecules may switch on pathways that result in prevention of oxidative damage or repair

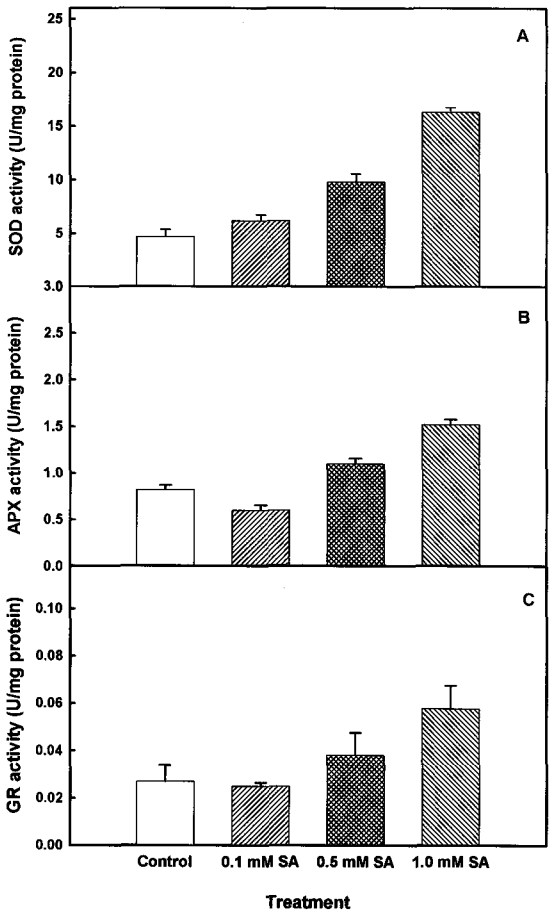

Fig. 5. Effect of SA on the activities of antioxidant enzymes in cucumber primary leaves exposed to different concentrations of SA.

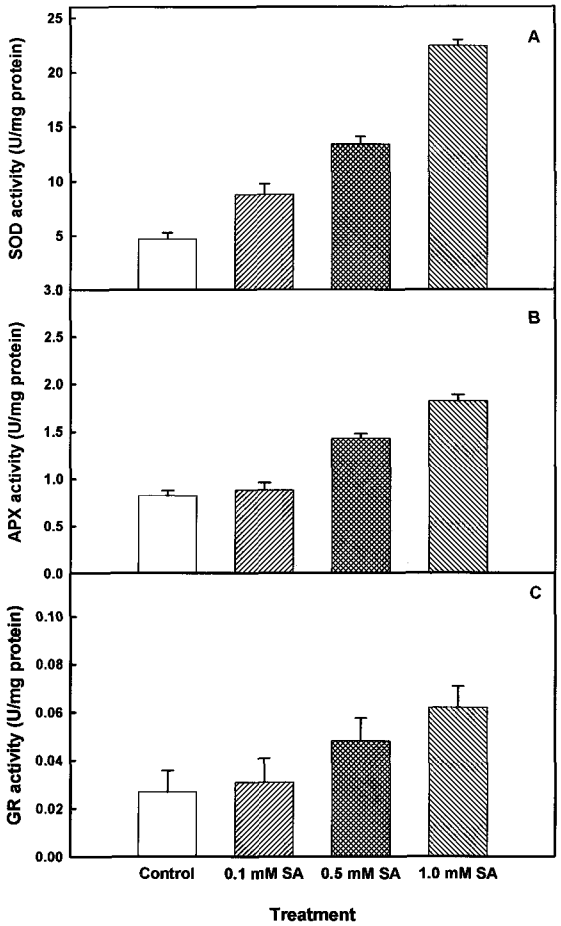

Fig. 6. Effect of SA on the activities of antioxidant enzymes in cucumber primary leaves exposed to UV-B and different concentrations of SA.

of that damage.

Most environmental stress are thought to result in the production of active oxygen species in plants, causing oxidative stress. The ability of higher plants to scavenge the toxic active oxygen seems to be a very important determinant of their tolerance to environmental stress. There are several antioxidant enzymes involved in the scavenging of active oxygen in plants, and the activities of same of these enzymes are known to increase upon exposure to oxidative stress[25]. The ascorbate-glutathione cycle is the most important antioxidant cycle in plants[26]. The first reactive oxygen species produced in plant cells is the superoxide radical, that is dismutate to H_2O_2 by superoxide dismutase (SOD). The H_2O_2 is reduced to H_2O by ascorbate peroxidase (APX). The ascorbate oxidized by APX is reduced by the reduced form of glutathione, and the glutathione is again reduced by glutathione reductase (GR). Under oxidative stress, superoxide radical increases and can stimulate the SOD activity. APX is considered a crucial component in the metabolic defense against oxidative stress. Thus higher APX activity was apparently associated with higher ascorbate biosynthesis capability[27]. In chloroplast, SOD, APX and GR are regarded as key enzymes of the scavenging pathway and UV-B irradiation is known to induce their activities in various plant species ; for example, SOD and APX in *Arabidopsis thaliana*[25], GR in *Triticum aestivum* [28].

There is increasing evidence that SA may be a signal that interacts with active oxygen species such as H_2O_2 during biotic and abiotic stresses[23]. Exogenous

SA can increase H_2O_2 levels in plant tissue, while H_2O_2 treatment stimulates SA biosynthesis. The growing tobacco plants on SA-supplemented media under oxidative stress induced H_2O_2 accumulation and affected antioxidant enzymes and metabolites, showing decreasing catalase and increasing certain ascorbate-glutathione cycle enzyme[6]. There is also evidence for interactions among SA, H_2O_2 levels and the induction of antioxidant enzymes for protection from UV-B damage. The selective enhancement of phenolic compounds with antioxidative ability in response to oxidative stress has also been observed in various plants including cucumber[29], tobacco[7] and *Arabidopsis*[8]. Therefore, according to the current results, the apparent role of SA was potentiating the stress response of the cucumber seedlings during oxidative stress generated by UV-B.

The effect of ABA and JA on UV-B tolerance was compared with that of SA to examine the physiological role in protecting cucumber plants from UV-B induced oxidative damage. The application of 1.0 mM ABA or JA showed similar tendency in the activities of antioxidant enzymes (Fig. 7). The maximal rise was also observed in the UV-B exposed leaf followed by the UV-B + ABA or UV-B + JA treatment. ABA and JA also induced some degree of UV-B tolerance, showing reduced damage in recovery from UV-B by these treatments. ABA and SA that were previously reported to have chemical and physiological similarites were similar to the effects caused by SA in physiological responses. SA, ABA and JA have been implicated as second messengers in signalling in response to a variety of stresses[30]. ABA is thought to induce thermotolerance and involve heat stress, suggesting that ABA result in prevention of oxidative damage or repair of that damage. JA also have been regarded to be a putative regulator of plant growth and development involved in chilling tolerance and wound response[31]. ABA and JA showed similar mode of action in UV-B stress, suggesting that they induce the same abundant proteins which may be important in acquired stress tolerance. Some evidence support that SA and ABA may mediate protection against, or repair of, oxidative stress.

Taken together, the data revealed that UV-B radiation affects cucumber yield, but some of the adverse

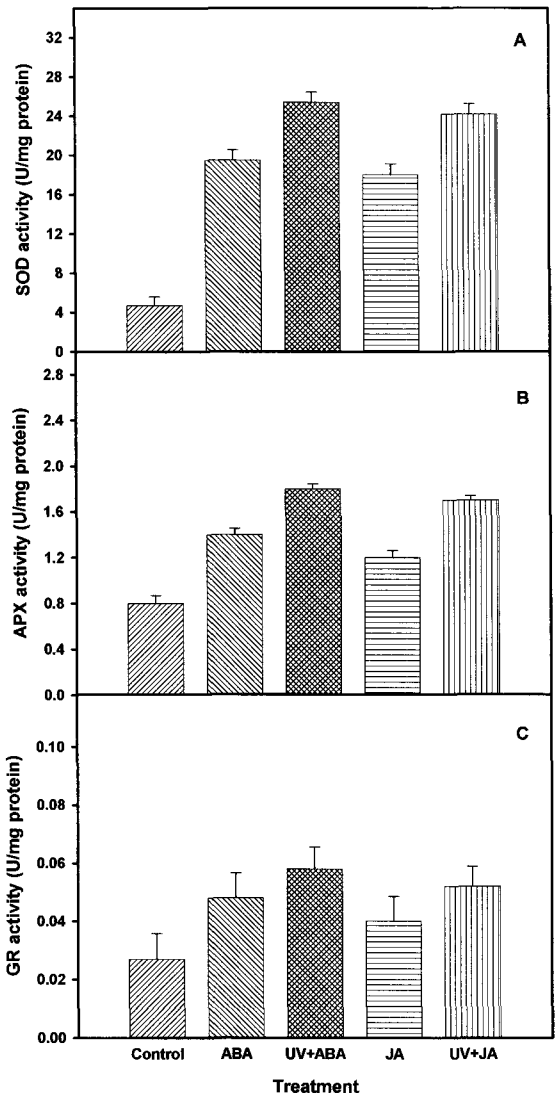

Fig. 7. Effect of ABA and JA on the activities of antioxidant enzymes in cucumber primary levaes exposed to UV-B and 1.0 mM ABA or JA.

effects of UV-B radiation can be partially ameliorated by SA. Thus SA may be involved in protection against UV-B induced oxidative stress. However, the relationship between these and other environmental factor is complex and little studied, although there are some data indicating that SA can modulate some of the harmful effects of heat, chilling and paraquat. Therefore, multifactorial experiments are required to know more about plant growth and physiological responses to environmental stressors.

Acknowledgements

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