Effect of salicylic acid spray on leaf ascorbate, proline and quercetin content in ultraviolet stressed Pepper seedlings

Kobra Mahdavian[*]

Department of Biology, Faculty of Science, Payame Noor University, Tehran 19395-3697, Iran
[*]**Corresponding author:**
(Received: 25/01/2017-Accepted: 26/09/2017)

Abstract:

The aim of the present study was to investigate the effects of SA on UV-A, UV-B, and UV-C stressed plants. Data showed that SA counteracted the UV harmful effects on pepper. Pepper (*Capsicum annuum* L.) seedlings sprayed with salicylic acid (SA) and treated with UV-A (320-390 nm), UV-B (312 nm), and UV-C (254 nm) of 6.1, 5.8, and 5.7 W m^{-2}, respectively. UV significantly increased contents of ascorbate, dehydro ascorbate, total ascorbate, proline, aldehyde, quercetin, electrolyte leakage and total ethylene. SA treatment moderated ascorbate, dehydro ascorbate, total ascorbate, aldehyde, electrolyte leakage and total ethylene increase in plants treated with UV. The quantity of proline and quercetin in plants that treated with UV and SA were significantly increased. Exogenous application (foliar spray) of SA counteracted the UV effects on pepper.

keywords: *Capsicum annuum*, Electrolyte, Ethylene, leakage, Lipid peroxidation.

Introduction:

UV radiation produces oxidative stress (Costa *et al.* 2002), which arises from the deleterious effects of active oxygen species (AOS) and react with lipids, pigment, proteins and nucleic acid (Dai *et al.*, 1997). Under conditions of normal healthy growth, plants possess a number of enzymatic and non-enzymatic detoxification mechanisms to efficiently scavenge either the AOS themselves or their secondary reaction products (Bartling *et al.*, 1993). Since flavonoids and phenolics absorb UV-B bands they represent a selective UV-B filter which protect plant tissue against harmful rays (Rozema *et al.*, 2002). Protective responses stimulated by UV-radiation include increase in production of UV-absorbing compounds and secondary compounds including hydroxyl cinnamic acid derivatives, phenylpropanoids, and flavonoids which effectively absorb the UV-radiation (Hofmann *et al.*, 2000; Skorska and Szwarc, 2007).

Transgenic plants showed an upregulation of the genes responsible for flavonoid (particularly, kaempferol and quercetin) biosynthesis under elevated UV-B conditions (Wang *et al.*, 2000; Ryan *et al.*, 2002). Studies have shown that UV radiation alters membranes. This can be seen by the increase in malondialdehyd concentration (MDA), reduced monogalagtosyl diacylglycerol (MGDG), as well as an increase in ethylene and ethane concentration (Dai *et al.*, 1997). Antioxidants such as ascorbic acid and glutathione (GSH) are involved in the neutralization of secondary products of AOS reactions (Conklin *et al.*, 2000).

SA as plant hormones has multifunctions in the plant, retards ethylene synthesis, interferes with membrane depolarization, photosynthesis, increases the content of chlorophyll (chl) as well as blocking wound response in soybean (Leslie and Romani, 1988; Zhao *et al.*, 1995). SA is a major phenylpropanoid compound that influences plant resistance against pathogens or other environmental stresses (Mahdavian *et al.*, 2007). There is also evidence that exogenous application of SA can alter the antioxidant capacity in plants (Mahdavian *et al.*, 2007).

However, information is not available on the effects of UV radiation and salicylic acid on the activated oxygen species metabolism and antioxidant activity in *Capsicum annuum*. This knowledge can supply information on the possible involvement of antioxidants as a defense against ROS generated by UV radiation and salicylic acid. Hence, the objective of this work was to investigate whether salicylic acid may act as protection to ameliorate the influence of UV stress in pepper plant and thereby increasing its UV tolerance. We tried to reveal those biochemical and physiological effects of SA pre-treatment which led to improved fitness of plants exposed to UV stress.

Materials and methods:

Growth conditions and cultivation: Seeds of *Capsicum annuum* L. were sown in plastic pots containing 1 kg of coarse sand and vermiculite (2:1, V/V). Plants were grown in the green house at 25/20 ℃ (day/night), with a 16h light/8h dark photoperiod for 35 days. Five-week-old pepper (*Capsicum annuum* L.)

plants grown in vermiculite in plant growth chamber were sprayed with 1.5 mM SA solutions once on completely expanded fourth leaves in early morning. A constant volume sprayed in all cases with a manual sprayer. Then the plants exposed to UV-A (320-390 nm), UV-B (312 nm), and UV-C (254 nm) irradiation with a density of 6.1, 5.8, and 5.7 W m^{-2}, respectively, (Leybold Didactic, Germany). UV-A, UV-B, and UV-C lamps purchased from the Philips (UV-tech England). Each pot treated with UV in their light period for 27 min per day for 14 d. The plants divided into four groups: 1) control, 2) plants treated with SA (1.5 mM), 3) plants that received UV-A, UV-B, or UV-C, 4) plants pre-treated with SA (1.5 mM) and then exposed to UV-radiation. All four replicates were used in each treatment.

HPLC analysis of quercetin: Four plants from each treatment group were chosen randomly for quercetin analysis. The frozen at -80 °C samples (100 mg) extracted with methanol: acetic acid: water (100:2:100, v/v) at room temperature for 1 h. The HPLC analysis were performed by injection of 20 µl of extract sample on a Lichrospher 100RP-18 (5 µm) column (250×4 mm), A non-linear gradient of acetonitrile was then run to elute the flavonoids (3 min at 10%, 1.5 min at 11.5, 9 mins at 14%, 2 mins at 19%, 9 mins at 22%, and 6 mins at 100% acetonitrile). Quercetin eluted at 12.5 in and the peak area was compared with the standard and detection at 355 nm (Greenberg *et al*., 1996).

Thiobarbituric acid reactive substances (TBARS): Three leaf tissues (0.2 gr) homogenized in 10 ml of 0.1% (w/v) trichloroacetic acid (TCA), and then centrifuged at 10,000 g for 15 minutes. One ml of supernatant then vortexed with 4 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid (TBA), and the solution was heated for 30 minutes at 95°C. The samples cooled on ice for 5 mins and recentrifuged for 10 minutes at 10000 g. Absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm for the MDA measurement (Heath and Packer, 1969), and at 455 nm for other aldehydes (Meirs *et al.,* 1992). For the MDA and aldehyds calculation, an extinction coefficient (ε) of 1.56×10^5 M^{-1} cm^{-1} used at 532 nm for MDA and an ε of 0.457×10^5 M^{-1} cm^{-1} used at 455 nm as the average of the ε obtained for five other aldehydes (propanal, butanal, hexanal, heptanal and propanal-dimethyl acetal).

Measurements of the content of ascorbic acid, dehydroascorbic acid and total ascorbate: Ascorbate and dehydro ascorbate determined as described by Mc de Pinto and Gara (1999). Simply 0.5 g of tissue homogenized in 10 ml metaphosphoric acid 5% and centrifuged for 15 minutes at 10,000g. 300 µl of supernatant used for the ASA assay and these solutions were added to the extract respectively: 750 µl potassium phosphate buffer and 300 µl distilled water 300 µl of supernatant was also used for the DHAS assay and the following solutions were added: 750 µl potassium phosphate buffer (pH=7.2) and 150 µl of 10 mM dithiothreitol, the mixture incubated at room temperature for 10 minutes, and then 150 µl of 0.5% N-ethylmaleimide added. Both samples vortexed and incubated at room temperature for 10 minutes. To each sample 600 µl of 10% (w/v) TCA, 600 µl of 44% (v/v) H_3PO_4, 600 µl of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 10 µl of 3% Fecl$_3$ added. After vortex-mixing, samples were incubated at 40 °C water bath for 20 minutes, and then samples were brought up and vortexed again and incubated at 40 °C water bath for 20 minutes. Absorbance of samples at 525 nm was recorded. A standard curve of ASA and DHAS used to calculate ASA and DHAS concentration.

Proline, membrane permeability and ethylene determination: Free proline was extracted, derivatized with acid ninhydrin and absorbance read according to Bates et al. (1973) method. Fresh leaf samples (about 0.1 g fresh weight, FW) were homogenized in 3 ml 3% sulfosalicylic acid and the homogenate was centrifuged at $1700 \times$ g for 10 min. A 0.5 ml aliquot of the supernatant was transferred to a test tube, 0.5 ml acetic acid and 1 ml acid ninhydrin were then added, and the mixture was boiled for one hour. The reaction was terminated in an ice bath. The reaction mixture was extracted with 1 ml toluene, mixed thoroughly by vortex. The optical density of the upper toluene phase was determined at a wavelength of 520 nm, using toluene as the blank, and L-proline as a standard. Membrane permeability of leaves were measured by electrolyte leakage (Dhindsa *et al*., 1981); leaves were cut and placed in a beaker containing distilled water and after 3 h at room temperature the conductivity of the solution measured. Ethylene production determined by GC (Kalantari, 1989).

Results and Discussion:

Plants exposed to UV-A, UV-B and UV-C showed significant increase in quercetin content of leaves. SA application increased quercetin content in plants exposed to UV (Figure 1). Our HPLC analysis of flavonoids showed increasing flavonoids may protect plants against oxidative stress, or prevent the penetration of destructive bands of UV light to the most sensitive tissue. In the present research, we studied the effect of SA in regulating UV induced oxidative stress in *Capsicum annuum* L. leaves. The flavonoids play many defensive roles in plants, and interception of UV-B by epidermal flavonoids often proposed as an adaptive mechanism preventing UV-B from reaching the mesophyll and affecting photosynthesis (Liu *et al*., 1995). Thus, the pepper plants may activate a defense mechanism against UV damages by increasing non-photosynthetic pigments. However, the antioxidant function of flavonoids is complex and depends on a variety of factors, including compartmentalization, redox potential and presence of double bands, glycosylation and hydroxylation (Bors *et al*., 1995; Rice *et al*., 1996; Cooper-Driver and Bhattacharya, 1998).

MDA and other aldehyde concentration of plants which exposed to UV-A, UV-B and UV-C increased
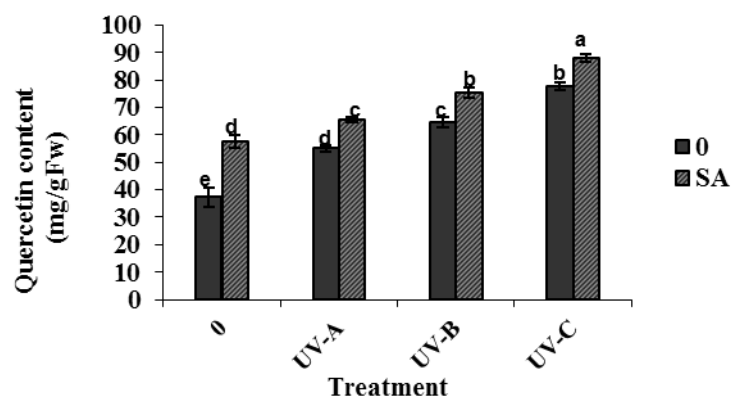
Figure 1- Changes in contents of quercetin [mg g⁻¹ Fw] of Pepper plants in response to UV-A, UV-B, and UV-C treatments in presence or absence of salicylic acid (SA). Means ± SE, means significantly different (LSD) at *P* = 5 % level are marked by different letters.
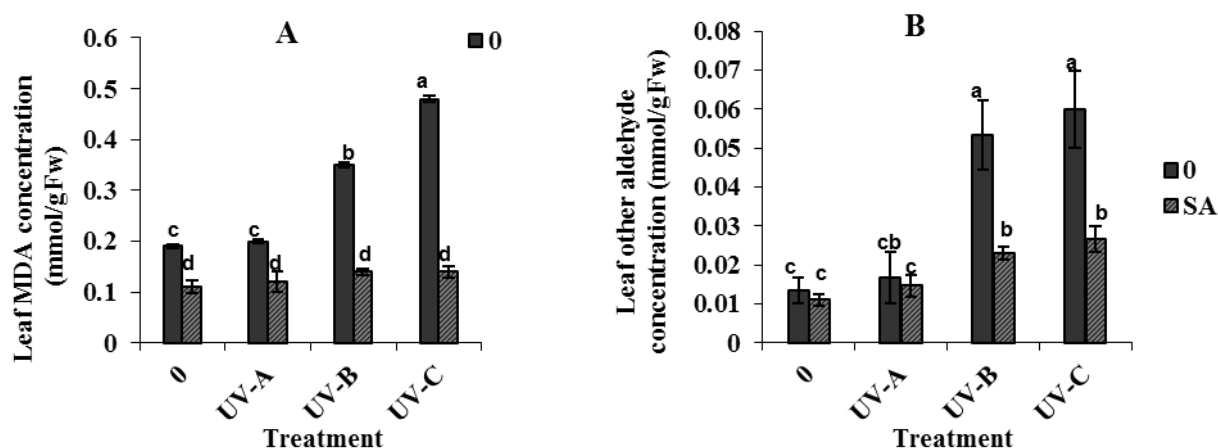

Figure 2- Changes in contents of (A) malone dealdehyde (MDA) and (B) other aldehyde [mMol g⁻¹ Fw] of Pepper plants in response to UV-A, UV-B, and UV-C treatments in presence or absence of salicylic acid (SA). Means ± SE, means significantly different (LSD) at *P* = 5 % level are marked by different letters.

significantly. SA treatment moderated the increase of MDA and other aldehyde concentration in plants treated with UV radiation (Figure 2). Oxidative damage can be detected by lipid peroxidation. Hydroxyl radicals and singlet oxygen can react with lipids and form lipid peroxy radicals and hydroperoxide (Blokhina *et al*., 2003; Hollosy, 2002). The peroxy radicals can abstract hydrogen from other unsaturated fatty acids, leading to a chain reaction of peroxidation. The peroxidation of membrane lipids leads to the breakdown of their structure and function (Hollosy, 2002; Yuan *et al*., 2000). The increase in TBARS content is more precisely an indicator of general UV-induced oxidative damage, due to the impairment of cell defense system (Costa *et al*., 2002; Barka *et al*., 2000). Indirect evidence from many experiments suggests that UV-B and UV-C exposure generates free radicals, which increase the lipid peroxidation and disruption of membrane integrity (Tripathi *et al*., 2017; Kramer *et al*., 1991). Ultraviolet is known to induce free radical formation and a consequent oxidative damage (Dietz *et*

al., 1999). Under UV treatment, an increase in MDA content indicated the oxidative stress in leaves. MDA content, however, was lower in leaves pretreated with SA. The enhancement of MDA production and subsequent lowering under SA treatment was further substantiated by the histochemical staining pattern of the leaves. The end products of lipid peroxidation are reactive aldehydes.

Leakage of electrolytes content of Pepper which was exposed to UV-A, UV-B and UV-C increased significantly. SA treatment moderated the increase of Leakage of electrolytes content in plants treated with UV radiation (Figure 3).

These findings indicate an important role of UV-B and UV-C radiation in quercetin synthesis. It seems that applied doses of UV radiation exert a state of stress, where limits of tolerance are exceeded and adaptive capacity is overtaxed, that possibly results in a disturbance of quercetin synthesis.

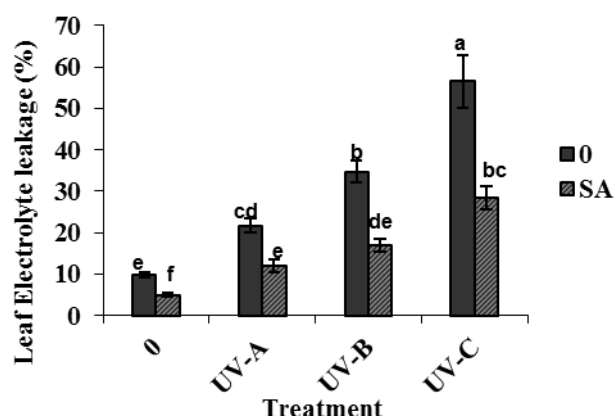Plants exposed to UV-B and UV-C showed significant increase in proline content of leaves. SA

Figure 3- Changes in contents of electrolyte leakage [%] of Pepper plants in response to UV-A, UV-B, and UV-C treatments in presence or absence of salicylic acid (SA). Means ± SE, means significantly different (LSD) at *P* = 5 % level are marked by different letters.
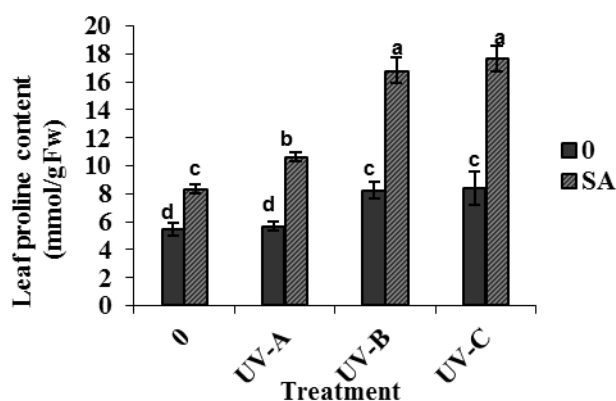

Figure 4- Changes in contents of proline [mMol g^{-1} Fw] of Pepper plants in response to UV-A, UV-B, and UV-C treatments in presence or absence of salicylic acid (SA). Means ± SE, means significantly different (LSD) at *P* = 5 % level are marked by different letters.

Figure 5- Changes in contents of ethylene [μl g^{-1}Fw] of Pepper plants in response to UV-A, UV-B, and UV-C treatments in presence or absence of salicylic acid (SA). Means ± SE, means significantly different (LSD) at *P* = 5 % level are marked by different letters.
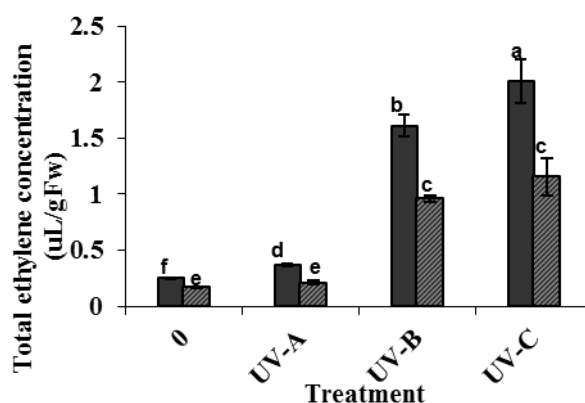
enhanced proline content in plants exposed to UV (Figure 4). Proline may be regarded as a scavenger of hydroxyl and singlet oxygen radicals (Smirnoff and Cumbes, 1989; Alia *et al*., 2001). Proline is known to be involved in alleviating cytosolic acidic associated with several stresses (Kurkdjian and Guern, 1989). The removal of excess H$^+$ occurring as a result of proline synthesis may have a positive effect on reduction of the
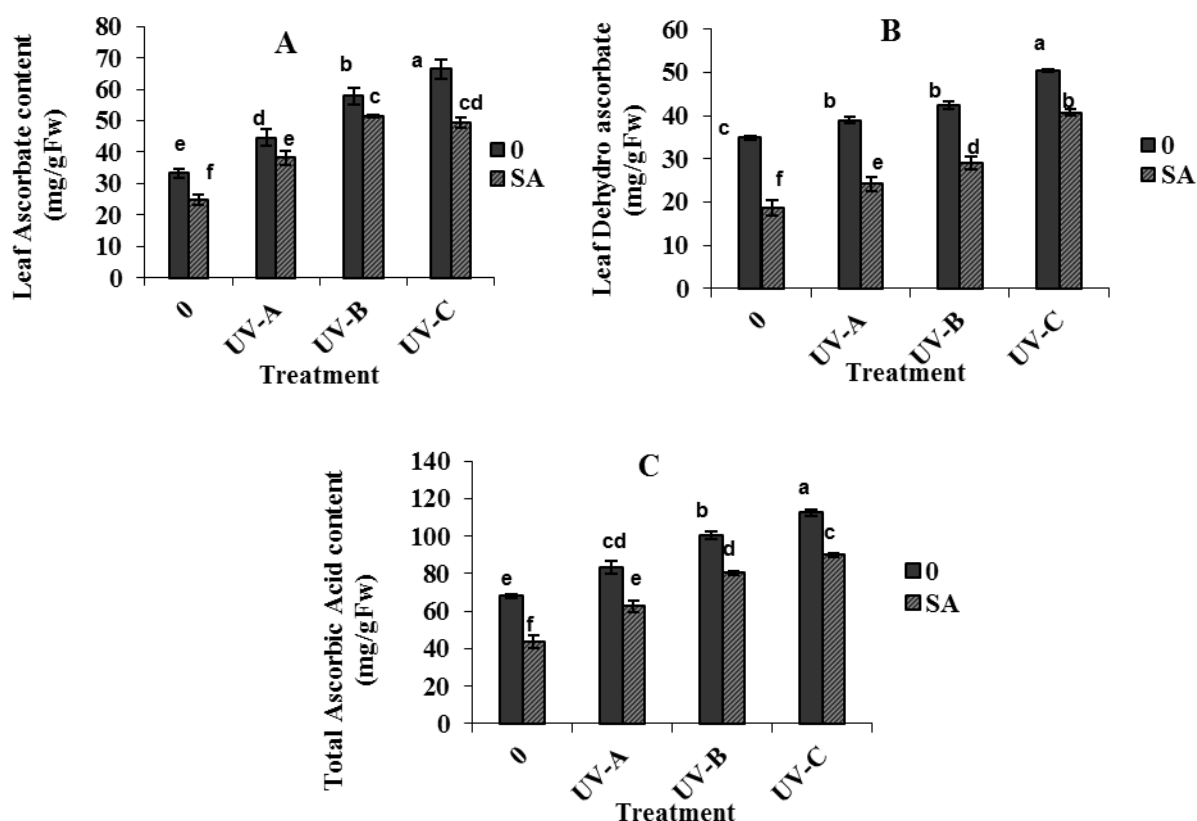
Figure 6- Changes in contents of ascorbate (A), dehydro ascorbate (B) and total ascorbate (C) [mg g^{-1}Fw] of Pepper plants in response to UV-A, UV-B, and UV-C treatments in presence or absence of salicylic acid (SA). Means ± SE, means significantly different (LSD) at _P_ = 5 % level are marked by different letters.

UV-B and UV-C induced damage. This may indicate that UV radiation induced proline accumulation which protects plants against UV radiation promoted peroxidation processes.

Plants exposed to UV-A, UV-B and UV-C showed significant increase of total ethylene concentration of leaves. SA application moderated the increase of total ethylene concentration in plants exposed to UV (Figure 5). SA is an effective inhibitor of ethylene biosynthesis (Romani _et al_., 1989). This effect seems to results from the inhibition of ethylene biosynthesis by a reduction in production of superoxide which is the mediator of the conversion reaction of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Vishwakarma _et al_., 2017; Wu and von Tiedemann, 2001).

Ascorbate, dehydro ascorbate and total ascorbate contents of plants which were exposed to UV-A, UV-B and UV-C increased significantly. SA treatment moderated ascorbate, dehydro ascorbate and total ascorbate increase by 0.7 time compared with the control (Figure 6). An ascorbic acid deficient Arabidopsis mutant was very sensitive to a range of environmental stresses, an observation that shows protective role in Arabidopsis foliar tissues for this molecule (Conklin _et al_., 1996). Ascorbic acid is postulated to maintain the stability of plant cell membranes against oxidant damage by scavenging cytotoxic free radicals (Akram _et al_., 2017). Salicylic acid (SA) is an important signal element and endogenous growth regulator involved in local and endemic disease resistance in plants (Enyedi _et al_., 1992).

Conclusion:

Results indicate that SA increases plant resistance against oxidative stress or photoprotection against UV radiation. In summary, it might be concluded that SA treatment of ultraviolet stressed pepper could stimulate their UV tolerance by activating oxygen species metabolism and antioxidant activity in _Capsicum annuum_.

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