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Original Article

Salicylic Acid and UV-B/C Radiation Effects on Growth and Physiological Traits of *Satureja hortensis* L.

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Abstract

Higher UV irradiation may affect the structure of ecosystems directly or indirectly. In the present work, *Satureja hortensis* L. defence mechanisms against UV stress was studied with or without salicylic acid (SA). For this aim, a factorial based experimental was conducted on completely randomized design with three UV treatments (control, UV-B and UV-C) as first factor and two SA levels (0 and 1 mM) as second factor. The results showed UV-B and C decreased shoot dry weight, plant height (PH), node number (NN), inter-node distance (IND), root length (RL), leaf area index (LAI) and chlorophyll content, but increased stem diameter (SD), leaf thickness (LTh), total flavonoid content (TFC), total phenolic content (TFC) and antioxidant activity. Plants treated with SA (1 mM) showed higher growth factors than non-treated one. It seems that SA can be used as an alternative substance against UV stress.

Keywords: salicylic acid, *Satureja hortensis* L., antioxidant assay, TFC, TPC

Abbreviations: Dry weight: DW, Fresh weight: FW, Leaf area index: LAI, Rehydration characteristic: RC, Salicylic acid: SA, Total flavonoid content: TFC, Total phenolic content: TPC, Ultra violate: UV, Antioxidant assay: AA

Introduction

Satureja hortensis L. belonging to Lamiaceae family is an annual herbaceous plant, known as Marzeh in Iran. Vegetative parts can be eaten raw or cooked in different nutritional regimes. In addition, *S. hortensis* is used as a traditional folk medicine and as an additive to improve the taste and scent of ayran and yogurt. Recent pharmacological studies have confirmed some medicinal properties of *S. hortensis* including anti-spasmodic, anti-diarrhea, antioxidant, sedative and anti-microbial effects (Gursoy *et al*., 2009). Therefore, the plant can be used as nutraceutical or food sources with medicinal properties.

It has been reported that UV treatments increase flavonoids and anthocyanins in plant tissues (Rozema *et al*., 2002; Zhang and Björn, 2009) and cause a broad range of morphological responses (Hopkins *et al*., 2002). There is evidence that continued depletion of ozone will lead to high penetration of ultraviolet-B/C rays to the Earth's surface (Xiong and Day, 2001). Approximately 10% of the solar rays that shines constantly on the Earth are ultraviolet radiation and are divided into three wavelength range: UV-C (200-280 nm, mostly absorbed by stratosphere), UV-B (280-315 nm, mostly absorbed by stratosphere) and UV-A (315-400 nm, partially absorbed by stratosphere) (Paul and Gwynn-Jones, 2003). Ultraviolet radiation in plants causes oxidative stresses, which are associated with damage of cellular components such as DNA, proteins, lipids and pigments (Hollosy, 2002).

Recently, scientists are interested to study the effects of UV on secondary metabolites enhancement. There is evidence that UV enhanced total phenols (Lee *et al*., 2014) and flavonoids (Zhang and Björn, 2009). Salicylic acid (SA) is a natural phenolic signalling molecule, which has shown an important role in plants when exposed to biotic and abiotic stress (Wang *et al*., 2010). The positive effects of SA against abiotic stresses have been reported with ozone, UV-B, heat, osmotic stress and heavy metals (Wang *et al*., 2010). NaCl and drought induced oxidative stresses are related with higher endogenous SA (Borsani *et al*., 2001; Németh *et al*., 2002). Exogenous SA application is involved in many physiological processes of plants, including stomatal closure, seed germination, fruit yield, glycolysis and photosynthesis processes (Cutt and Klessig, 1992; Arfan *et al*., 2007), production of antioxidant enzymes, tocopherols, anthocyanins and other phenolic compounds (Hayat *et al*., 2010).

The aim of the present study was to determine morphologic- and physiological changes induced by UV-B and C on *S. hortensis*. In addition, SA was used as an exogenous agent to overcome reduction of morphological change by UV irradiation. In other words, the purpose of the experiment was to enhance total phenol and total flavonoid amounts without

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any negative effect on morphological traits. For this means, antioxidant activity, total phenol, total flavonoid were also determined.

Materials and Methods

Plant material

Seeds of *Satureja hortensis* L. were purchased from Pakanbazr Company, Esfahan, Iran. Then they were cultured in plastic pots (six cm diameter) (Fig. 1), in a mixed media containing soil, leaf mold and sand (1: 1: 1) (pH = 7.16 and EC = 0.33 $ds.m^{-2}$). Seedlings were grown in a greenhouse at 25 ± 2 °C day/ 18 ± 2 °C night temperature, under 16/8h photoperiod with 11,000 Lux at 60% relative humidity. After four weeks, the uniform growing seedlings were used for different treatments.

UV and SA treatments

Uniform 4-weeks old seedlings were subjected to UV and SA treatments. The used treatments in this study were as following: control, salicylic acid (SA) (1 mM), UV-B (162 $j/m^2/day$), UV-B (162 $j/m^2/day$) + SA (1 mM), UV-C (18 $j/m^2/day$), UV-C (18 $j/m^2/day$) + SA (1 mM). The seedlings were irradiated during 18 days with two days interval, for 15 and 5 min per day for UV-B and UV-C respectively. UV-B radiation was treated by a 20 W fluorescent tube (F20T8 BLB Preheat), whereas UV-C was treated by a 20 W fluorescent tube (Sylvania-Ultra-Violate G 20 W, Japan). For combined treatments, SA was used 10 min after UV irradiation. Afterwards, the treated seedlings were transferred to plastic pots (15 cm diameter) and were grown for 60 days until flowering stage and then harvested for further studies.

Selected growth and morphological treats

For each treatment, the plants were evaluated in terms of growth characteristics, including plant height, stem diameter, leaf area index, leaf thickness, internode distance, root length, fresh and dry weight of roots and shoots, and number of nodes.

Total number of nodes (TNN) was calculated as:

$$TNN = (NPLB \times NOLB) + NPMB$$

Where NPLB represents nodes per lateral branches, NOLB is the number of lateral branches, NPMB represents nodes per main branches.

Chlorophyll and carotenoids determinations

The chlorophyll and carotenoid content of the leaves were evaluated by previously established extraction method (Arnon, 1949). Therefore, 0.5 g of fresh leaves were mixed with 2.5 ml distilled water, crashed by porcelain mortar to obtain a uniform mass. The uniform mass was poured into clean vials and added distilled water to obtain 50 ml of final volume. Then 0.5 ml of prepared extraction was taken and mixed with 2.5 ml of 80% acetone and then was centrifuged at 7,000 rpm for 10 min. The optical density of taken supernatant was read at 470, 465 and 663 nm using a spectrophotometer (Unico 2100-UV Single Beam UV/Vis) and finally, pigments' quantity was calculated using the formula reported by Gross (1991).

Fig. 1. A) Treatment arrangement in plastic pots within the experiment; B) plants at flowering stage

Preparation of extractions

Dried leaf material (200 mg) of each treatment were extracted in separate vials with 10 ml diethyl ether and kept for 24 h, as described in the literature (Vieira *et al*., 2001). In order to prevent the evaporation of diethyl ether, the vials were kept closed and the extraction was performed in a cold room. After 24 h, the extracts were poured into clean vials and the leaves were rinsed with another 5 ml diethyl ether, which were added to the initial extracts. The diethyl ether was evaporated in an extraction cabinet until complete dryness. After adding 5 ml of 80% aqueous methanol solution, the extracts were filtered into clean vials. This prepared solution was used to TFC, TPC and antioxidant evaluation.

Measurement of TPC

Total phenol in the methanol extracts was determined colorimetrically using Folin-Ciocalteu reagent, as established by (Slinkard and Singleton, 1977) 30 µl of prepared extract with 600 µl of Folin-Ciocalteu reagent (10%), mixed with 90 µl of distilled water and let to remain for 10 min. After that, 480 µl of sodium carbonate was added and the mixture kipped in dark place for 1.5 to 2 h. Color changing in the extract was determined colorimetrically at a wavelength of 765 nm by spectrophotometer. Gallic acid (GAE) was used as a standard, and results were expressed as mg gallic acid equivalents per 1 g dry matter basis.

Measurement of TFC

The TFC were determined by a colorimetric assay (Shin *et al*., 2007) 500 µl of the extracts described above were added to a 15 ml tube containing 2 mL of deionized water. Then 150 µl of 5% sodium nitrite was added to this mixture and remain at room temperature for 5 min. After that, 300 µl of 10% aluminium chloride ($AlCl_3.6\ H_2O$) was added and allowed to further react for 6 min, then 1 ml of 1 $mol.l^{-1}$ sodium hydroxide was added. Distilled water was added to bring the final volume of the mixture to 5 ml. The absorbance of the solution was measured immediately at 510 nm. The results were expressed as catechin (CAT) equivalents using a standard curve prepared from authentic catechin.

Antioxidant assay

Free radical 2, 2-dipheynyl-1-picrylhydrazyl (DPPH) was used for antioxidant assay. In order to determine free radical scavenging activity, aqueous methanol solution described above was used in this section. Free radical scavenging activity (RSA) was measured according to the principle of Nakajima *et al*. (2004) with some modifications reported by Chiou *et al*. (2007). Fifty microliters of the prepared extracts were added to 950 µl of 6×10^{-5} mol l^{-1} (free radical, 95%, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in methanol. The mixtures were

shaken and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Methanol was used as an experimental control. The percent of reduction of DPPH or RSA was calculated by using the following formula:

$$DPPH\% = \frac{(Abscontrol)_{t=30\min} - (Abssample)_{t=30\min}}{(Abscontrol)_{t=30\min}} \times 100$$

Where, Abs sample is the absorbance at 517 nm of the DPPH in the presence of the sample and Abs control is the absorbance of DPPH solution without extract.

Rehydration characteristic (RC)

RC is an index which reflects indirect information about cell wall structure. In order to establish RC data, 0.5 g of dried leaves of each treatment were weighed and placed in separate Petri dishes. Then, 5 ml of water were added and allowed to be absorbed for 24h. After that, materials were drained for 10 min to remove surface water and were weighted again. RC was measured by flowering formula:

$$RC = \frac{(rehydratedweight)_{t=24h} - (dryweight)}{dryweight} \times 100$$

Data analysis

Data analyses were carried out with the SAS 9.1 package statistical software for windows. Duncan multiple range test was used to compare the significance of differences among means of treatments.

Results and Discussion

Selected growth and morphological treats

The mean inter-nodal distance (MIL) ($P > 0.05$) and shoot length ($P > 0.01$) of *S. hortensis* were found to vary among the different treatments (Table 1). MIL ranged from 2.64 to 3.28 cm, with maximum and minimum values recorded in SA (3.28 cm) and UV-C (2.64 cm) treated plants, respectively. The shoot length (ShL) varied from 34.91 cm in UV-C- to 39.91 cm in SA treated plants. In UV + SA treated plants MIL and ShL increased as high as control. Root length (RL) in the treated plants was not significantly different from the control. Similar results with MIL and ShL have been reported in cotton (Kakani *et al*., 2003) under UV-B irradiation. It has been reported that UV radiated soybean had lower MIL and ShL than control (Zhang *et al*., 2014). Phytohormons reduction and ethylene enhancement are the main reasons for decreasing plant normal growth (Teramura, 1983).

The differences in fresh and dry weight of roots and shoots among the various treatments were statistically significant ($P < 0.05$). The highest shoot, root fresh and dry weight were recorded in plants treated with SA and control (Table 1), whereas plants treated with UV-C and UV-B produced lower fresh and dry weight respectively. Similar results have been reported in wheat (He *et al*., 2011) and beans (Singh and Singh, 2011) under UV-B radiation. These evaluated growth factors, shoot and root fresh and dry weight, were also improved by combined treatments, SA with UV-B and UV-C. It seems that SA acts as moderating agent when plants are exposed to UV irradiation. The positive role of SA in plants resistance to abiotic stresses have been reported with ozone, UV-B, heat, osmotic stress and heavy metals (Wang *et al*., 2010). It has been reported that SA increased the dry weight of roots, shoots of corn and soybeans (Khan *et al*., 2003). UV classified as abiotic stress reduce plant growth and decline phyto-hormones (biosynthesis and transport) viz. IAA and GA (Krizek *et al*., 1998).

Analysis of variance showed significant differences ($P < 0.01$) in leaf thickness, stem diameter and node number between treatments. Compared with control, plants that were exposed to UV-B and UV-C irradiation significantly increased leaf thickness, stem diameter and nods number. In SA treated plants, these indices were lower than those treated with UV (Table 1). Leaf thickness has been reported in UV-irradiated plants (Santos *et al*., 2004). Photosynthetic tissues are more sensitive than non-photosynthetic one against UV irradiation, which may be due to free radical production when oxidative stress occurs. Leaf thickness in photosynthetic tissues has been known as a protective mechanism against UV irradiation (Krizek *et al*., 1998). Also stem diameter has been reported as another important morphological index in *Artemisia annua* L when treated with UV-B (Rai *et al*., 2011).

The differences in leaf area index (LAI) between various treatments were statistically significant ($P < 0.01$). LAI in plants exposed to UV-B (694.5 mm^2) and UV-C (686.2 mm^2) irradiation was significantly reduced compared to control (854.5 mm^2); the reduction was not

Table 1. Effect of different treatments on morphological factors at the flowering stage of *Satureja hortensis* L.

	ShFW (g)	ShDW (g)	RFW (g)	RDW (g)	IND (cm)	RL (cm)	ShL (cm)	LAI (mm2)	TNN	LT (mm)	SD (mm)
control	18.13ab	2.82a	6.16 ab	0.83 a	3.01 ab	26.12ab	38.16ab	854.50 a	72.11c	0.34c	1.91 c
SA	19.33a	2.88 a	7.39 a	0.87a	3.28 a	28.01 a	39.91 a	842.08 a	71.70c	0.36bc	2.00bc
UV-B	15.22d	2.52ab	5.39bc	0.54bc	2.89 b	25.00ab	35.77 cd	694.50cd	80.33b	0.37ab	2.11 ab
UV-B+ SA	17.40bc	2.73 a	5.52 bc	0.58b	3.01 ab	25.67ab	37.01 bc	777.30b	80.84b	0.38 ab	2.13 a
UV-C	12.63e	2.22b	4.32c	0.44 c	2.64 b	23.71 b	34.91d	686.28 d	90.87a	0.39 a	2.13 ab
UV-C + SA	16.34cd	2.77a	5.55 bc	0.48 bc	2.89 b	24.74ab	36.26cd	749.30bc	81.26b	0.38 ab	2.17 a
LSD	1.32	0.43	1.25	0.12	0.36	3.56	1.77	55.00	7.06	0.02	0.12
Significant level	**	*	**	**	*	ns	**	**	**	**	**

Note: Different letters between treatments denote significant differences (Duncan test, $P < 0.05$); *, ** and ns: Significant at $P \leq 0.01$, 0.05 and not significant respectively

Shoot fresh Weight (ShFW), Shoot dry weight (ShDW), Root Fresh Weight (RFW), Root dry weight (RDW), Inter-nod distance (IND), Root length (RL), Shoot length (shL), Leaf area index (LAI), Total nod number (TNN), Leaf thickness (LT), Stem diameter (SD)

significant in plants treated with SA (Table 1). Similar change has been reported in *Artemisia annua* L. (Rai *et al*., 2011). UV irradiation caused a reduction in leaf area, as a photomorphogenic response to reduce the leaf tissues damages (Jansen *et al*., 1998). Reduction in LAI may be due to UV radiation-induced changes in the rate and extent of cell division and expansion (Hopkins *et al*., 2002). Lower LAI is one of the main reasons in biomass reduction and photosynthetic capacity (Barnes *et al*., 1993).

Chlorophyll and carotenoid content

Analysis of variance showed significant differences ($P < 0.01$) in total chlorophyll content between treatments. UV-B and UV-C irradiated plants showed a significant decrease in the chlorophyll content. However, there was no significant difference in carotenoid content of UV+SA-treated plants (248.01 to 296.53 mg. g^{-1} FW) in comparison with control (263.19 mg. g-1 FW). Chlorophyll reduction in UV+SA treated plants was less than for plants treated only by UV (Table 2).

The results of the experiments revealed that chlorophyll and carotenoid content increased in SA-treated plants. Significant reduction of photosynthetic pigments in leaves of pepper irradiated with UV-B and UV-C has been shown previously (Raskin, 1992). Similarly, on *Barleria obtusa* UV decreased chlorophyll content (Murali *et al*., 1988). Reduction in chlorophyll content can be due to non-enzymatic inhibition of chlorophyll synthesis (Agrawal, 1992), ethylene enhancement (Zhang and Kirkham, 1996) and/or photo oxygenation (Gao *et al*., 2003).

Also SA spray had an incremental effect on leaf chlorophyll and carotenoid content (Türkylmaz *et al*., 2005). The main reason for increasing photosynthetic pigments in plants treated with salicylic acid can be due to the stimulative effect of SA on chlorophyll synthesis (Khurana and Maheshwari, 1980). Carotenoids increased in some species an adaptive response to reduce the effects of UV radiation, because the carotenoid caused by the scattering of excited energy xanthophyll cycle and thus are protected by photosynthetic apparatus (Inzé and Van Montagu, 2002).

Total phenol content (TPC)

Total phenol content was significantly different among various treatments ($P < 0.01$) (Table 2). The results of the analysis showed that the amount of TPC in UV- and SA-treated plants was significantly higher than control. High amount was obtained in UV-C treatment (970.71 µg. g-1 DW) followed by UV-C+SA (869.29 µg. g-1 DW). UV-B and UV-B+SA treated plants had 853.1 and 773.5 of TPC. UV radiation effects on the metabolism of secondary compounds and acts as elicitor on propanoid polyphenols phytochemicals that flavonoids, simple phenols, tannins and lignin are also synthesized. Phenolic compounds protect cells against free radicals (Omaye *et al*., 1979). According to the results, SA also increased the TPC (Table 2). Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenyl propanoid pathway, leading to the conversion of phenylalanine to *trans* phenyl propanoids synamic acid, which is the precursor of active compounds like flavonoids. SA stimulates the expression and activity of these enzymes. Also, SA increased Chalcone isomerase depended compounds, and acts as an absorber of UV (Li *et al*., 2007). Various studies on the plants treated with UV-B radiation significantly increased the amount of TPC in *Lactuca sativa* (Lee *et al*., 2014). Phenolic compounds protect cells against oxidative stress (Rozema *et al*., 2002). The phenolic compounds of aromatic amino acids (phenylalanine and tyrosine) are produced through the path of phenylpropanoids to decrease UV- induced destruction effects (Bieza and Lois, 2001).

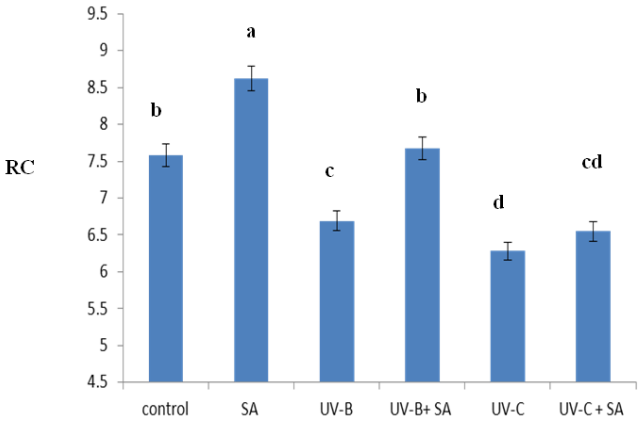

Fig. 2. Rehydration characteristic (RC) affected by various treatments in *Satureja hortensis* L. Data represent means of three replicates represented by standard division. Different letters between treatments denote significant differences (Duncan test, $P < 0.05$)

Table 2. Effect of treatments on physiological properties in the leaf extract of *S. hortensis* L.

	Carotenoids (mg.g^{-1} FW)	Total chlorophyll (mg.g^{-1} FW)	Total phenol (µg.g^{-1} DW)	Total flavonoids (µg.g^{-1}DW)	Total antioxidants (% DPPH)
Control	263.19 ab	8.44 a	663.10e	255.79b	38.71 b
SA	285.19 ab	8.64 a	719.76de	279.96 ab	41.61 ab
UV-B	295.63 a	7.66 bc	853.10bc	308.50 ab	42.13 ab
UV-B+ SA	296.53 a	8.31 ab	773.57cd	354.96 ab	42.42 ab
UV-C	248.01 ab	7.03 c	970.71a	367.46a	47.65 ab
UV-C + SA	289.66 b	7.31 c	869.29b	306.83 ab	54.42 a
LSD	42.62	0.65	93.25	69.29	13.43
Significant level	Ns	**	**	ns	ns

Note: Different letters between treatments denote significant differences (Duncan test, $P < 0.05$); *, ** and ns: Significant at $P \leq 0.01$, 0.05 and not significant respectively

Total flavonoid (TFC) and antioxidant activity (AA)

Analysis of variance showed significant differences ($P < 0.01$) in TFC and AA among treatments. Total flavonoids (μg.g-1 DW) among different treatments were various as following amounts: control (255.79), SA (279.96), UV-B (308.50), UV-B+SA (354.96), UV-C (367.46), UV-C+SA (306.83). DPPH scavenging activity was 47.65 and 54.42 (μg.g-1 DW) in UV-C and UV-C+SA-treated plants, higher than control (38.71). Plants treated with UV radiation increased the amount of TFC and AA (Table 2). SA increased TFC and AA comparing with control plants. Similar results have been reported with total flavonoids by other researchers in *Pisum sativum* L. (Choudhary and Agrawal, 2014) and *Lolium perenne* (Comont *et al*., 2013). Under UV radiation some plants are able to produce and accumulate UV-absorbing compounds suh as flavonoid, which are related to the expression of their corresponding genes. Flavonoids, with the highest number of hydroxyl groups, can participate in free radicals (Dawar *et al*., 1998). High expression in PAL enzyme is related with flavonoid biosynthesis (Wang *et al*., 2006). It has been reported that flavonoids may play a protective role as an antioxidant against harmful free radicals created by UV radiation (Hilal *et al*., 2004). Chalcon synthase, with an essential role in the biosynthesis of flavonoids, is lso increased by UV radiation (Sakihama *et al*., 2002).

Rehydration characteristic (RC)

RC was affected by various treatments (Fig. 2). The results showed that dry material of plants treated with UV-C and UV-B had less water absorption, whereas SA-treated plants had more water absorption. RC in dried material strongly reflects cell wall situation. Thus, UV-B and UV-C has been reported as a cell wall distraction agent, mostly corresponding to oxidative damage of cellular components such as DNA, proteins, lipids and pigments (Hollosy, 2002), therefore the potential for RC is decreased in these treated plants. The regulated use of SA and cell wall strengthening could account for explain why in combined (UV+SA) treatments, RC was improved.

Conclusions

UV radiation increased some phytochemicals such as phenolic and flavonoids and caused changes within morphological attributes, decrease of inter-nod distance and leaf size. It seems that SA can be used as an effective factor to increase the resistance of plants against UV stress. The use of UV-B and C can be effective elicitors for enhancing phenolic compound in *S. hortensis*. It seems that SA can reduce UV induced negative morphological effects, without significant reduction in TPC and TFC. However, further studies are necessary to fully understand how SA increased *S. hortensis* plant resistance under UV radiation.

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