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The Effect of Different Irrigation Cycles and Salicylic Acid on Certain Quantitative and Qualitative Traits of *Viola tricolor* L.

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ABSTRACT

The influence of salicylic acid on environmental stress tolerance has been known since many years, but owing to the impact of drought stress on green space plants in Iran, for the first time, was utilized this hormone on *viola cornuta* Rebel in Shiraz. The experiment was factorial in a completely randomized design, with twelve treatments and four replications in each treatment. Three levels of irrigation cycle [0, 3, and 5 daily] were used for this seasonal flower and four levels of salicylic acid [0, 0.5, 0.75, and 1 mM] were sprayed three times on the violets. The results represented salicylic acid at rates of [0.5, 0.75, and 1 mM], which, in interaction with the levels of the irrigation cycle [3, and 5 daily], can reduce the drought effect. Plant height, leaf area, leaf width, Chlorophyll a and b, proline content, and RWC increased and only the electrolyte leakage diminished. In the traits leaf length and carotenoid content, cannot be seen a noticeable difference; but, generally from the above findings, it can be concluded that salicylic acid declines water-stress effect on the majority of the pansy traits.

Keywords: *Viola tricolor*, salicylic acid, irrigation cycle, drought stress.

Abbreviations: SA: salicylic acid; IRC: irrigation cycle; IRC2: every two days of the irrigation cycle; IRC3: every three days of the irrigation cycle; IRC5: every five days of the irrigation cycle; RWC: relative water content; EL%: electrolyte leakage.

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INTRODUCTION

Viola tricolor L., as seasonal flower, provides beautiful colorful spots for early spring in the Iran metropolitan cities (Golestani et al., 2013). For instance, the Shiraz (one of the most important cities in Iran) Park Organization, reported that the greatest amount of seasonal flower plantation among all the flowers of the city was for the pansy, with 60,000 m² of plantation. Meanwhile, diminishing water resources all over the world is a barrier. The Iran Meteorology Bureau reported that the 46-year average rainfall in Shiraz was 319.8 mm, while in the past year, it was only 131 mm. Approximately, one-third of the world's arable land suffers from inadequate water supplies (Kramer et al., 1980). Because the violet contains polysaccharides, salicylic acid derivatives, catechins, and coumarins, its employment as a medicinal plant is ancient (Czygan et al., 2002). Salicylic acid (*ortho* hydroxybenzoic acid) belongs to plant phenolic compounds and is an endogenous growth hormone that participates in the regulation of physiological process in plants, such as flowering, thermogenicity (heat-producing) in thermogenic plants (Raskin et al., 1992), seed germination, fruit yield, glycolysis (Klessig et al., 1994), response to environmental stresses like pathogen attack, disease-resistance (Chen et al., 1995), water, salinity, and cold stress (Kenji et al., 2014) and heat stress (Qinghua et al., 2006). While *viola tricolor* contains a noticeable amount of salicylic acid derivatives, positive results in this experiment can a sign that will allow the application of salicylic acid (SA) in tough conditions for other plant species with lower levels of SA. To the best of our knowledge, this is the first report in which SA has been applied in an urban green space for defending against drought stress.

MATERIALS AND METHODS

Plant Materials and Experimental Conditions

This experiment was conducted at the Shiraz Azad University Horticultural Science Department (29°36' N and 52°31' E, at an

elevation of 1,545 m). The violet seeds from the *viola cornuta* Rebel. variety were cultivated in pots that had been filled with sand and were irrigated daily. When the seedlings reached the three leaves-stage, we transferred each four sets of seedling to five lit plastic pots, containing a 2: 1: 1 (v/v) mixture of sand, clay, and decomposed farmyard manure. The pots were kept in a greenhouse and the plants were subjected to natural day/night conditions with 12-hour photoperiods, light intensities of 2800-3000 lux, air temperatures of 18-22°C at night and 22-26°C during the day, and a relative humidity of 65 ± 5 percent. After four-leaves stage, we utilized daily irrigation cycle and thereafter SA foliar application once a month; and we gathered leaves in the third-month after four-leaves stage, which were immediately frozen in liquid nitrogen and were stored at -80°C for subsequent analysis from a day later.

Experimental Design and Data Analysis

Our experiment was a factorial in a completely randomized design, with twelve treatments, including IRC (IRC₂ as the control treatment, IRC₃, and IRC₅) and SA (0, 0.5, 0.75, and 1 mM) and four replicates in each treatment, and four samples (plants) in each replication. Data were analyzed using the SAS software (version 9.1.3) and the means were compared using the least significant difference (LSD) test at $p < 0.05$.

Plant Height, Leaf Length, Leaf Weight and Leaf Area Index

Plant height was measured with a ruler; the leaf length and width were measured with calipers, and the leaf area index was measured with the leaf area meter (model Delta T Devices).

Relative Water Content (%)

We determined RWC by the Barrs and Weatherley (1962) method. After weighing 1-cm²-diameter leaf discs (FW), floating for 24 hours in deionized distilled water, weighing Turgid weight (TW), dehydrating at 80°C for four hours, and weighting them (DW), the RWC was defined by the following formula:

$$RWC = (FW - DW) - (TW - DW) 100$$

Membrane permeability (Electrolyte Leakage)

A 1-cm-diameter sample after washing by deionized water to remove surface-adhered electrolytes, was immersed in 10 ml of double-distilled water at 25°C on a rotary shaker (100 rpm) and Electrical conductivity of the bathing solution (L_t) was determined after 24 hours. Thereafter sample was autoclaved at 120 °C for 20 minutes and a last conductivity reading (L_o) was obtained upon equilibration at 25 °C. EL% was determined by the following equation (Lutts et al., 1996):

$$EL\% = (L_t / L_o)100$$

Chlorophyll a, b, and Carotenoid Content

For chlorophyll calculation, the Hiscox et al. (1979) method and the equation which had been proven from Arnon's method (1949) was applied.

Proline Content

According to method applied by Bates et al. (1973), proline was calculated by the spectrophotometer at a wavelength of 520 nm.

RESULTS

The results show that by increasing the irrigation cycle, all the experimented plant factors diminished, and EL% and proline increased.

Salicylic acid can increase all the treatments, and for leaf length, leaf width, and leaf area, the best SA concentrations were 0.75 and 1 mM, although in leaf length and leaf width there is not a difference between these concentrations and 0.5 mM. The best concentration for EL%, proline, and chlorophyll a was 1 mM. For plant height, RWC, chlorophyll b, and carotenoid content, the highest amount was 0.75 mM, although in plant height between 0.5 and 0.75 mM, in chlorophyll b between 0.75 and 1 mM, and in carotenoid content between 0.5, 0.75, and 1 mM, a significant difference cannot be seen. With respect to the interaction between

IRC and SA for the leaf width, 0.5 and 0.75 mM in

Variance analysis of some quantitative and qualitative traits of *Viola tricolor* L.

		MS.									
S.O.V	df	Leaf length	Leaf width	Leaf area	Plant height	EL%	RWC%	Proline	Chlorophyll a	Chlorophyll b	Carotenoid
IR	2	722.93**	361.33 **	328794.77**	526.89**	0.17**	1275.49**	1406.97**	0.02**	0.001**	0.001**
SA	3	26.01*	25.13**	60248.74**	19.18**	0.02**	2099.29**	1084.09**	0.01**	0.0001**	0.0001**
IR*SA	6	8.15	10.12**	9330.65**	6.89*	0.001**	146.74*	18.85*	0.002**	0.0007**	0.00001
Error	36	8.12	2.36	2473.10	3.14	0.0005	70.25	6.23	0.00002	0.0001	0.00001
CV (%)	-	8.32	7.19	9.27	8.66	6.85	12.25	3.26	2.64	2.60	9.48

*and** significant at the 5% and 1% levels of probability, respectively

IRC₂, 1 mM in IRC₃, and all the SA doses in IRC₅, have a significant effect on this trait. With respect to leaf area, all SA concentrations in IRC₂, 0.75, and 1mM in

IRC ₂	IRC ₃	IRC ₅	IRC ₂	IRC ₃	IRC ₅	IRC ₂	IRC ₃	IRC ₅			
Leaf length (mm)			leaf width (mm)			leaf area (mm)			plant height (cm)		
43 ^a	35 ^b	30 ^c	28 ^a	23 ^b	18 ^c	74 ^a	60 ^b	46 ^c	28 ^a	21 ^b	17 ^c
EL (ms)			RWC (%)			Proline (mg/g)					
0.25 ^c	0.33 ^b	0.45 ^a	79 ^a	78 ^a	62 ^b	72 ^c	82 ^b	89 ^a			
Chlorophyll a (mg/g)			Chlorophyll b (mg/g)			Carotenoid (mg/g)					
0.22 ^a	0.18 ^b	0.14 ^c	0.052 ^a	0.048 ^b	0.35 ^c	0.047 ^a	0.042 ^b	0.032 ^c			

The effect of irrigation cycle on some quantitative and qualitative traits of *Viola tricolor* L.

For each average numbers with the same letter according to Duncan's multiple range tests at 5% probability level are not statistically different than each other

SA ₀	SA _{0.5}	SA _{0.75}	SA ₁	SA ₀	SA _{0.5}	SA _{0.75}	SA ₁	SA ₀	SA _{0.5}	SA _{0.75}	SA ₁				
Leaf length (mm)				leaf width (mm)				leaf area (mm)				plant height (cm)			
32.25 ^b	34.65 ^a	35.50 ^a	35.45 ^a	19.5 ^b	21.8 ^a	22.5 ^a	22.3 ^a	50 ^c	58 ^b	64 ^a	67 ^a	19 ^c	21 ^{ab}	22 ^a	20.25 ^{bc}
EL% (ms)				RWC (%)				Proline (mg/g)							
0.28 ^d	0.32 ^c	0.36 ^b	0.39 ^a	58 ^d	69 ^c	88 ^a	73 ^b	69 ^d	78 ^c	84 ^b	90 ^a				
Chlorophyll a (mg/g)				Chlorophyll b (mg/g)				Carotenoid (mg/g)							
0.145 ^d	0.155 ^c	0.195 ^b	0.205 ^a	0.039 ^c	0.042 ^b	0.047 ^a	0.046 ^a	0.0355 ^b	0.0394 ^a	0.042 ^a	0.0405 ^a				

The effect of salicylic acid on some quantitative and qualitative traits of *Viola tricolor* L.

For each average numbers with the same letter are not statistically different than each other according to Duncan's multiple range tests at 5% probability level. IRC₃, and 0.75 mM in IRC₅ had a clear difference with the control condition. With regard to the plant height, solely 0.5 and 1 mM in IRC₂, and 0.75 mM in IRC₅ have a significant impact on this factor.

Decreasing plant water causes the EL% to increase and in between 1 mM, SA is the best dose for the declining EL%. With regard to RWC, this subject is reversed and the best SA concentration is 0.75 mM. With respect to proline, all the SA concentrations are effective, and the highest influence is a consequence of 1 mM of SA. With respect to Chlorophyll a and b, although approximately all the SA doses can

lead to a rise in IRC₃ and IRC₅, only all the SA concentrations in IRC₃ for Chlorophyll a, and 0.75 and 1 mM in IRC₅ for Chlorophyll a and Chlorophyll b can illustrate a clear difference in comparison to the control condition. As for the carotenoid, we cannot see a significant difference among all the SA levels in various irrigation cycles in comparison to the control treatment.

DISCUSSION AND CONCLUSION
When drought stress rises, decreasing all the experimented factors, and increasing EL% and proline are a common reaction for plant rescue. Rising EL% causes cell membrane

IRC SA	IRC ₂ IRC ₃ IRC ₅			IRC ₂ IRC ₃ IRC ₅			IRC ₂ IRC ₃ IRC ₅					
	Leaf length	leaf width		leaf area		plant height						
0	39.94 ^{ab}	34.67 ^c	28.76 ^{de}	24.12 ^b	19.85 ^{de}	13.81 ^g	591.75 ^{cd}	481.00 ^{efg}	364.00 ^h	24.50 ^b	18.25 ^{cde}	14.00 ^g
0.5	38.99 ^b	30.17 ^{de}	27.06 ^e	28.01 ^a	20.95 ^{cd}	16.15 ^f	683.25 ^b	522.50 ^{de}	406.75 ^{gh}	27.25 ^a	19.75 ^{cd}	15.75 ^{efg}
0.75	43.02 ^{ab}	34.71 ^c	28.01 ^e	28.03 ^a	20.49 ^{de}	18.72 ^{de}	714.00 ^b	600.50 ^{cd}	495.50 ^{ef}	27.00 ^{ab}	20.75 ^c	18.00 ^{cde}
1	43.77 ^a	32.87 ^{cd}	28.66 ^{de}	24.12 ^b	23.02 ^{bc}	18.50 ^e	846.25 ^a	647.50 ^{bc}	422.25 ^{gh}	28.50 ^a	17.25 ^{def}	14.75 ^{fg}
	EL%			RWC			Proline					
0												
0.5	0.26 ^c	0.38 ^b	0.47 ^a		62.10 ^{de}	50.83 ^{ef}	47.67 ^f		55.38 ^h	66.91 ^f	71.66 ^e	
0.75	0.26 ^c	0.30 ^c	0.46 ^a		69.14 ^{cd}	74.85 ^{bc}	48.09 ^f		60.99 ^g	74.06 ^e	83.93 ^c	
1	0.20 ^d	0.30 ^c	0.41 ^b		86.39 ^{ab}	89.19 ^a	79.63 ^{abc}		70.96 ^e	84.54 ^c	89.88 ^b	
	0.19 ^d	0.26 ^c	0.37 ^b		76.72 ^{abc}	79.32 ^{abc}	57.03 ^{def}		78.39 ^d	84.84 ^c	94.78 ^a	
	Chlorophyll a			Chlorophyll b			Carotenoid					
0												
0.5	0.20 ^a	0.12 ^c	0.09 ^d		0.05 ^a	0.043 ^{ab}	0.02 ^c		0.04 ^{ab}	0.039 ^{abc}	0.025 ^c	
0.75	0.20 ^a	0.15 ^b	0.09 ^d		0.05 ^a	0.042 ^{ab}	0.032 ^{bc}		0.046 ^{ab}	0.042 ^{ab}	0.030 ^{bc}	
1	0.20 ^a	0.21 ^a	0.15 ^b		0.05 ^a	0.049 ^a	0.038 ^{ab}		0.048 ^a	0.042 ^{ab}	0.034 ^{abc}	
	0.20 ^a	0.20 ^a	0.19 ^a		0.05 ^a	0.049 ^a	0.039 ^{ab}		0.045 ^{ab}	0.039 ^{abc}	0.033 ^{abc}	

The effect of salicylic acid and irrigation cycle on Leaf length, leaf width, Leaf area, Plant height, EL%, RWC%, Proline and pigments

For each average numbers with the same letter are not statistically different than each other according to Duncan's multiple range tests at 5% probability level.

destruction, diminishing RWC, and decreasing leaf area respectively. Parsons (1982) mentions to avoid excessive transpiration, the leaf area will decrease and for achieving to this, leaf width and leaf length has to be decreased. The consequence of this issue is the decline of leaf pigments, including chlorophyll A, B, and the carotenoid content, and then the declining photosynthetic rate. By decreasing the photosynthetic rate, the amount of growth and plant height will diminish. At this time, the plant will increase proline in self-defense. Rhizopoulou et al., (1990) mentions to an enhancement in leaf proline content during the summer drought period in four *Phrygana* species owing to energy and nitrogen consumption, which stored in proline for resistance against stress. A lot of reports can confirm the influence of a water-deficit on

dropping growth factors, and rising proline and EL%. For instance Riaz et al., (2013) in *Tagetes erecta* L. about leaf area, leaf width, leaf length, plant height, RWC, chlorophyll A, B, and carotenoid, Yamada et al., (2005) in *Petunia hybrida* regarding proline, Jungklang et al., (2017) in *Curcuma alismatifolia* about plant height, RWC, EL%, and proline described the destructive effect of drought stress.

The enhanced RWC level and the diminished EL quantity under the rise of SA have been asserted in lettuce (Agami, 2013) and in *Dianthus caryophyllus* cv. liberty (Yazdy Zadeh et al., 2012). EL% illustrates cell membrane injury and drought stress destroys the membrane permeability (Agami, 2013). It is clear that when the cell membrane is destroyed, in addition to ions, cell water will leave the membrane. Enhancing proline production by SA (Delavary et al., 2010; El Tayeb et al., 2010) has complicated impacts, including protection from plasma cellular membranes when stress is experienced (Santoro et al., 1992). Proline drops membrane injury (Patel et al., 2011) by managing energy as a reductant anabolic pathways needs

(Shetty et al., 2004) as well as antioxidant activities, and stimulating phenolic and antioxidant response pathways (Smirnoff et al., 1989; Reddy et al., 1991). In addition, while applying SA and inducing proline production as osmoregulation, the amount of cell content will increase and then the water potential will decrease (Ball et al., 2002); therefore RWC will be preserved under stress.

Low turgor pressure under the waterless condition caused cell enlargement to diminish and as a result the plant height and all the growth factors decreased (Manivannan et al., 2007). Cell division, enlargement, and differentiation are the main processes that determine the quality and the quantity of plant growth; these factors are affected by environmental stresses like water stress (Patel and Golakia, 1988). SA can maintain plant height, leaf width and leaf area by certain actions, such as the accumulation of auxin and cytokinin (Shakirova, 2007), increasing photosynthesis for producing energy, rising proline production for transferring energy and preserving cell water. As for SA ineffectiveness on the leaf length, Borsani et al., (2001) concluded that SA efficiency concerns various reasons like species, application procedures, development stages, and SA dose.

Growth reduction and a decrease in the size of the leaf will diminish the rate of photosynthesis (Ruker et al., 1995). Senescence is a sign of a waterless condition and during senescence, the chloroplast structure is gradually disassembled by chlorophyllase and proteases, and after that the chlorophyll levels will decline due to the degradation and the disorganization of the grana lamellar stacks of the chloroplast (Paliyath et al., 2008). Rising Chlorophyll a and b, during SA application under drought stress, leads to an increase in photosynthesis and delaying senescence in the *Viola tricolor*. With respect to carotenoid, Barman mentions the mango insignificant difference of SA treatment from the control condition indicates that SA did not hamper the synthesis of the carotenoid

pigments. During the ripening (senescence) phase under ethylene production, chlorophyll loss and carotenoid synthesis rise (Schofield et al., 2008). Carotenoids protect the photosynthetic apparatus from the reactive oxygen species that are produced under stress (Siefermann-Harms, 1987; Young, 1991).

Although synthesized SA is not an organic substance, some of its characteristics like its safety of use, cheap price (the sole plant hormone that was extracted from the willow tree and was used as a drug many years ago was SA (Raskin et al., 1990)), and its ability of resistance against many environmental stresses allows us to apply it in a waterless condition. In addition, utilizing the noxious material against pests and diseases, which are a side-effect of drought stress (Tubby et al., 2010) will decline and consequently we can assert that the use of SA is in line with organic agriculture in regions with harsh conditions.

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