Adv. Hort. Sci., 2016 30(3): 151-157 DOI: 10.13128/ahs-20277

Salicylic acid at different plant growth stages affects secondary metabolites and phisico-chemical parameters of greenhouse tomato

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Key words: antioxidant activity, flavonoids, *Solanum lycopersicum*, total phenolics, yield components.

Abstract: Most of the researches on Salicylic acid (SA) have focused on postharvest application or acquiring stress resistance, while studies on its effect on plant growth, secondary metabolites and fruit quality are limited. SA as foliar application (0, 150, 300 and 450 mg/L) at different plant growth stages on fruit yield, secondary metabolites and quality features of tomato (*Solanum lycopersicum* L. cv. Kardelen) under greenhouse conditions were evaluated. The highest fruit yield per plant (about 1.3-fold greater than control) was obtained from 300 mg/L SA when applied three weeks after fruit set. Comparing to control plants, the highest fruit firmness, 10 days prolonged storability, highest total phenolics (22.6 mg gallic acid equivalent per 100 g FW); and highest antioxidant activity (65.11) were observed when 450 mg/L SA applied at fruiting stage and 3 weeks later. An increasing pattern in ascorbic acid content was observed with increasing SA concentration irrespective to application time. The same concentration effect was observed in flavonoid content when plants treated at 3 weeks after fruiting. The highest effect of flavonoids on antioxidant activity was calculated using Pearson correlation (r=0.82). SA concentrations greater than 450 mg/L showed significant adverse effects on all measured traits. The effect of exogenous SA on tomato plant depends on the developmental stage and SA concentrations tested. Improved fruit quality factors may happen in a certain concentration range, while over that may have negative or adverse effect.

1. Introduction

Tomato as one of the most widely produced and consumed 'vegetable' in the world (Heuvelink, 2005) contains high levels of antioxidant active compounds such as vitamin C, polyphenlos and carotenoids (Tommonaro *et al.*, 2012).

Salicylic acid (SA) has been the focus of intensive research due to its role in plant defense mechanisms and response to abiotic stresses (Rivas-San Vicente and Plasencia, 2011). Besides, it is stated that SA plays a crucial role in physiological and biochemical processes during the entire lifespan of the plant (Rivas-San Vicente and Plasencia, 2011). SA as an endogenous plant growth regulator controls a large variety of physiological processes: from regulatory signal in plants mediating defense against pathogens, to ethylene biosynthesis, action and inhibition. It is also involved in plant responses to abiotic stress conditions such as salt and osmotic stresses (Khalil, 2014). Exogenous application of SA also results in many different changes in plant physiological processes and reactions such as prevention of ethylene production (Khan *et al.*, 2003); increases in plant height, number of branches, number of leaves (Saharkhiz *et al.*, 2011) and antioxidant activity (Ananieva *et al.*, 2004).

Most of the researches on SA have focused on mediating local and systemic plant defense and resistance to biotic and abiotic stresses (Atkinson and Urwin, 2012), while studies on its effect on physiological, biochemical and quality features of fruit are limited (Ali *et al.*, 2014). Since plant growth, development and the level of bioactive compounds especially antioxidant active substances depend on the cultivar, and by agronomic and environmental conditions (Tommonaro *et al.*, 2012), the aim of this work was to study the influences of SA on growth, fruit quality attributes including fruit firmness and storability, vitamin C, antioxidant activity, total phenolic, flavonoids and yield of tomato under greenhouse conditions.

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Received for publication 1 May 2016
Accepted for publication 9 August 2016

2. Materials and Methods

Plant material and experimental design

The tomato (*Solanum lycopersicum* cv. Kardelen) seeds were obtained from Gento Seeds® Co., Turkey. Six weeks old seedlings having 4-5 leaves were transplanted into a 500 m^2 polycarbonate greenhouse silt-loam soil (Greenhouse Research Center, College of Agriculture, Shiraz University, Shiraz, Iran) at 50 cm double rows and 150 cm between rows spacing (density of 2 plants/m^2). The greenhouse conditions were set to temperature of 25±2°C and relative humidity of 60-70% during the entire study. Soil was already fertilized according to the soil test results and certified lab recommendations for growing greenhouse tomato (Papadopoulos, 1991).

Treatments consisted of SA (Merck Millipore Corporation, Germany) foliar application at four concentrations (0, 150, 300, and 450 mg/L) at different growth stages (transplant establishment, onset of flowering, fruit set, 3 weeks after fruit set) and their all possible combinations (Table 1). The experiment was arranged in a completely randomized design with three replications. Each sample for analyses consisted of three fruits (per plant) of four plants for each replicate per treatment.

Measurement methods

Fruits were harvested at mature red stage based on the "Color Classification Requirement in United States Standards for Grades of Fresh Tomatoes" chart, published by USDA.

Tomato produces fruits on clusters. Fruits of two first clusters were considered as yield and expressed as Kg/plant. Fruit firmness was measured as penetration force on the fruit flesh (over the fruit locules) using Force Gauge, FG-5005 (Lutron Electronic Enterprise Co. Taipei, Taiwan) with a probe diameter of 8 mm. The average values obtained for each fruit was calculated and expressed as Newton. Fruit storability was measured as the number of days from keeping red fruits in a storage at 12±1°C and 80% relative humidity to starting fruit shrinkage or lose their shiny appearance.

Vitamin C quantification was performed according to the method described by the AOAC (1984) and results were expressed as mg ascorbic acid per 100 grams of fruit DW.

Total phenolic content (TPC) was determined with the Folin-Ciocalteau reagent using the method of Spanos and Wrolstad (1990) and the results of three replicates were expressed as mg gallic acid equivalent per 100 grams of fruit DW (mg GAE/100g DW).

Table 1 - Effect of salicylic acid foliar application at different tomato plant phonological stages on some fruit characteristics

Measured trait	Salicylic acid (mg/l)	Application time										
		Establishment	Flowering	Fruiting	3 weeks after fruiting	Establishment + Flowering	Establishment + Fruiting	Establishment + 3 weeks after fruiting	Flowering + Fruiting	Flowering + 3 weeks after frui-ting	Fruiting + 3 weeks after fruiting	All 4 times
Fruit firmness (N)	150	9.97±0.47 no	10.76±0.59 mn	12.59±0.13 kl	12.20±0.21 lm	12.33±0.14 lm	12.62±0.24 kl	12.27±0.05 lm	12.67±0.36 kl	10.89±0.64 mn	13.49±0.99 j-l	13.94±0.20 i-k
	300	13.96±0.35 i-k	14.69±0.68 g-j	16.14±0.82 e-g	14.51±0.39 g-i	15.07±0.24 f-j	15.56±0.12 e-h	14±0.50 h-k	15.33±0.28 f-i	14.38±0.07 g-i	16.11±0.52 e-g	16.10±0.65 e-g
	450	16.19±0.83 e-g	16.28±0.87 e-g	19.92±0.78 a	16.95±0.21 c-e	16.23±0.40 e-g	18.28±0.31 bc	16.45±0.37 ef	18.86±0.12 ab	16.60±0.32 d-f	18.89±1.17 ab	18.10±0.54 b-d
	Control	9.07±0.51 o										
Shelf life (days at 12°C)	150	21.00±0.57 d	21.66±0.33 b-d	21.66±0.33 b-d	22.00±1.0 b-d	21.66±0.23 b-d	21.66±0.66 b-d	21.66±0.31 b-d	21.33±0.33 cd	21.66±0.41 b-d	22±0.57 b-d	22±0 b-d
	300	21.66±0.33 b-d	22±0.57 b-d	22±0.51 b-d	22.33±0.33 b-d	22.00±0.54 b-d	22.33±0.33 b-d	22.33±0.13 b-d	22.33±0.34 b-d	22±0.02 b-d	22.33±0.33 b-d	22.33±0.33 b-d
	450	22.33±0.66 b-d	22±0.57 b-d	22.33±0.88 b-d	24.66±1.33 a	21.83±0.44 b-d	22.33±0.33 b-d	22.66±0.88 bc	22.66±0.23 bc	23±0.57 b	23±0.57 b	22.33±0.33 b-d
	Control	17.00±0.00 e										
Vitamin C (mg/100 g dw)	150	13.88±0.35 pq	13.99±0.35 pq	14 39±0.42 o-q	18.28±0.26 l-o	15.42±0.81 n-q	16.92±0.62 m-p	19.12±0.51 l-n	14.74±0.39 o-q	20.37±0.92 k-m	21.03±1.24 j-l	21.87±1.90 i-l
	300	23.56±0.22 h-k	24.91±1.63 h-j	27.15±1.25 e-h	28.47±1.63 b-g	25.10±0.68 h-g	25.13±0.13 h-g	26.01±0.48 f-h	27.30±0.51 d-h	27.52±1.53 d-h	27.81±1.06 c-g	29.35±2.28 b-f
	450	31.11±0.79 a-e	31.62±0.89 a-c	32.47±0.12 ab	32.50±0.09 ab	31.18±0.73 a-e	31.26±0.72 a-d	31.95±0.92 ab	31.73±0.92 a-c	32.25±0.38 ab	34.34±2.07 a	32.50±0.84 ab
	Control	11.82±0.24 q										
Total phenolics (mg GAE/100 g dw)	150	14.19±0.09 l	14.51±0.16 k	15.60±0.25 i	16.14±0.14 gh	15.12±0.10 j	15.18±0.10 j	15.71±0.05 i	14.59±0.23 k	15.70±0.20 i	15.84±0.11 hi	16.26±0.22 g
	300	19.66±0.17 f	19.73±0.14 f	20.13±0.12 e	20.24±0.06 e	20.04±0.06 ef	20.12±0.065 e	20.13±0.06 e	19.99±0.12 ef	20.22±0.03 e	20.26±0.05 e	21.45±0.06 d
	450	21.47±0.03 d	21.71±0.29 b-d	22.64±0.09 a	22.67±0.12 a	21.54±0.07 cd	21.51±0.09 cd	21.59±0.11 cd	21.49±0.02 cd	21.88±0.18 bc	21.73±0.19 b-d	22.01±0.06 b
	Control	10.84±0.10 m										
Flavonoids (mg GAE/100 g dw)	150	1.05±0.08 n	1.06±0.08 n	1.06±0.01 n	1.09±0.03 kl	1.09±0.08 kl	1.07±0.03 mn	1.09±0.08 lm	1.08±0.03 lm	1.08±0.01 lm	1.08±0.09 lm	1.09±0.06 j-l
	300	1.09±0.06 j-l	1.13±0.04 i-k	1.11±0.07 h-j	1.14±0.01 b-e	1.09±0.06 j-l	1.09±0.09 j-l	1.12±0.06 g-i	1.10±0.05 j-l	1.12±0.03 f-h	1.14±0.09 d-g	1.09±0.10 j-l
	450	1.14±0.06 d-g	1.14±0.06 c-f	1.13±0.01 e-g	1.18±0.05 a	1.14±0.07 c-f	1.14±0.05 c-f	1.16±0.06 bc	1.14±0.01 d-g	1.15±0.07 b-d	1.16±0.08 ab	1.14±0.08 c-f
	Control	0.99±0.07 o										

Averages±SD for each measured trait with the same letters showing no significant differences using LSD test at p<0.05.
Control plants did not receive any SA at any time.

Total antioxidant activity (TAA) was measured using DPPH (2,2-diphenil-1-picrylhydrazyl) (Merck Millipore Corporation, Germany) assay as described by Patras *et al.* (2009) at the absorbance of 517 nm using micro plate reader (Epoch, Germany). TAA was calculated according to the following equation:

$$TAA = [1- (A_{sample\ at\ 517nm} / A_{control\ at\ 517nm})] \times 100$$

Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric assay based on the formation of a complex flavonoid-aluminum, having a maximum absorbance at 510 nm (Toor and Savage, 2005) and results expressed as mg gallic acid equivalent per 100 gram of fruit DW (mg GAE/100 g DW).

Statistics

Data were analyzed using SAS 9.1 statistical software (SAS Institute Inc., Cary, NC, USA). Means were compared using LSD test at $p \leq 0.05$. Pearson correlation statistical method was used to determine the correlation between secondary metabolites and antioxidant activity.

3. Results and Discussion

Our preliminary tests on different concentrations of SA application showed significant adverse effects on all measured traits when concentration was higher than 450 mg/l (data not shown). This could be due to the nature of SA that acts as a plant growth regulator. It is stated that the responses to SA are highly concentration dependent, so that moderate doses of SA improve features such as antioxidant status and induce stress resistance, while higher concentrations trigger a hypersensitive cell death pathway (Tounekti *et al.*, 2013).

Fruit yield

Fruits of the first two clusters were evaluated as yield. The greatest yield was obtained from 300 mg/l SA applied at three weeks after fruit set (Fig. 1). This was 1.26 fold greater than control. The stimulatory effect of SA on flowering regulation which has been well known for a long time (Raskin, 1992; Rivas-San Vicente and Plasencia, 2011) would eventually affect total number of fruits (Ondrašek *et al.*, 2007) and enhance efficiency in fruit production. Previously, increased yield of strawberry (Aghaeifard *et al.*, 2015) and tomato (Javaheri *et al.*, 2012) has been related to promoted cell division and cell enlargement due to SA (Hayat *et al.*, 2010) through its influence on other plant hormones such as auxin,

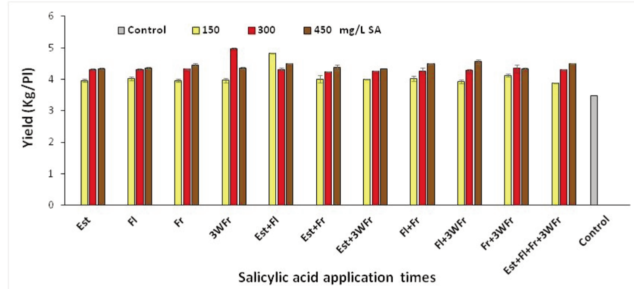

Fig. 1 - Effect of salicylic acid (SA) foliar application at different tomato plant phenological stages on yield (two first clusters) at greenhouse condition. Transplant establishment (Est), flowering (Fl), fruiting (Fr), three weeks after fruiting (3WFr).

cytokinin and ABA balances (Shakirova, 2007) and enhanced net photosynthetic rate, internal CO_2 concentration and water use efficiency (Fariduddin *et al.*, 2003). It is reported that in non-thermogenic plants such as tobacco, SA levels increase 5- and 2-fold in their leaves at the initiation of or during transition to flowering, respectively (Abreu and Munné-Bosch, 2009).

Fruit firmness

Firmness is an important physical parameter for postharvest storage, transportation and monitoring the fruit ripening process. SA and its derivatives are widely in use to enhance fruits postharvest life by enhancing fruit firmness during storage (Wang *et al.*, 2006). In our experiment, SA significantly affected fruit firmness. The firmness became 2.9 times higher than the control group when 450 mg/L SA was applied at fruiting stage. Plants treated with 150 mg/L SA at establishment stage showed the lowest fruit firmness as equal to control plants (Table 1). A 2-fold greater tomato fruit skin thickness due to 0.01M SA application comparing to control has been previously reported (Javaheri *et al.*, 2012). Metabolic reactions respiration, ethylene production (Aktas *et al.*, 2012) breakdown of cell wall and activation of enzymes involved therein are key events in fruit ripening and softening (Prasanna *et al.*, 2007). These metabolic activities can be harmful to maintain fruit quality. There are reports that SA could prevent the activity of such enzymes, while affect the swelling of cells in a manner that results in firmer fruit (Zhang *et al.*, 2003; Prasanna *et al.*, 2007). SA prevents fruit softening. Shafiee *et al.* (2010) have cited several reports indicating that rapid softening of fruits during ripening was simultaneous with rapid decrease in endogenous SA of fruits. Increased firmness in climacteric fruits due to pre-harvest SA spray has been attributed to the role of SA in preventing cell wall and membrane degrading enzymes (polygalacturonase, lipoxygenase, cellulase, pectinemethylesterase), ethylene production (Zhang *et al.*, 2003) and reduced hydrolysis of soluble starch and therefore higher firmness (Tareen *et al.*, 2012 b), while in non-climacteric fruits such as “Flame seedless” grape it has been attributed to the role of SA in preventing decay (Khalil, 2014).

Fruit storability

The greatest fruit storability period was observed in 450 mg/l SA when applied at 3 weeks after fruiting (Table 1). This was over 10 days more than control plants. Aghdam *et al.* (2014) attributed longer storability and higher chilling resistance of detached tomato fruits treated with SA to increased endogenous proline content. Lowered ethylene biosynthesis has been also considered to be the main cause of prolonged storability due to regulatory potential of exogenous SA on fruit ripening of green mature tomato fruits (Kant *et al.*, 2013). Parallel to this, SA can activate the alternative respiration pathway in many plant tissues which results in a lower respiratory rate and delay in the climacteric peak (Raskin, 1992). Srivastava and Dwivedi (2000) stated that the concentration of SA determines the extent to which these effects actualize. Reduced the quality loss during storage due to SA were previously reported for tomato (Ding *et al.*, 2001) and sweet peppers (Fung *et al.*, 2004).

Vitamin C

Human diet consists of about 91% of ascorbic acid coming from fruits and vegetables (Tareen *et al.*, 2012 a). An increasing pattern of ascorbic acid content was observed with increasing SA concentration irrespective to application time (Table 1). The greatest ascorbic acid content was obtained from 450 mg/L SA. In most cases, this increase was about 3 times than control plants. Some researches indicated that the treatment of tomatoes (Javaheri *et al.*, 2012; Kalarani *et al.*, 2002) and strawberry (Aghaeifard *et al.*, 2015) with SA caused them to acquire higher levels of ascorbic acid comparing to control plants. SA can activate ascorbate peroxidase, which is the precursor to ascorbic acid in fruits and prevents vitamin C from being destroyed in cells and therefore causes the accumulation of ascorbic acid in the fruit (Wiśniewska and Chełkowski, 1999).

Total Phenolics

Phenolic compounds as secondary plant metabolites are synthesized by all plants and responsible for the flavor and color of fruit products (Jeong *et al.*, 2008). The highest amount of phenolic compounds was observed in plants treated with 450 mg/L SA during the fruiting stage and three weeks after the fruiting (Table 1). In those treatments, a 2.9 times higher phenolic compounds content than control plants was observed. Although Aghdam *et al.* (2012) reported no significant effect of SA application on total phenolics content of mature green tomato fruits, our results were similar to reports on sweet cherry (Valero *et al.*, 2011) and grapes (Ranjbaran *et al.*, 2011; Khalil, 2014), which all concluded SA application induced greater total phenolics and other secondary metabolites with antioxidant properties (Ranjbaran *et al.*, 2011). Previously, the application time of SA on sweet cherry at three fruit developmental stages (pit hardening, initial color changes and onset of ripening) increased fruit weight and led to higher concentration of total phenolics and total anthocyanins, as well as higher antioxidant activity (Giménez *et al.*, 2014).

Antioxidant activity

It is well known that the positive effect on health associated with tomato consumption is exerted by the pool of antioxidants, with noticeable synergistic effects (Tommonaro *et al.*, 2012). The highest antioxidant activity was observed in 450 mg/L SA treatment when applied on fruiting stage plus three weeks after fruiting. This was 1.84 times greater than the control, which had the least antioxidant activity. An increasing pattern in antioxidant activity was observed with increasing SA concentration, irrespective to application time (Fig. 2). The same pattern was previously found in orange (Huang *et al.*, 2008 b) and pears (Cao *et al.*, 2006). Increased total antioxidant activity of strawberry due to SA has been previously reported (Aghaeifard *et al.*, 2015). Regular applications of salicylic acid at different stages of plant growth and fruit development can increase the antioxidant activity

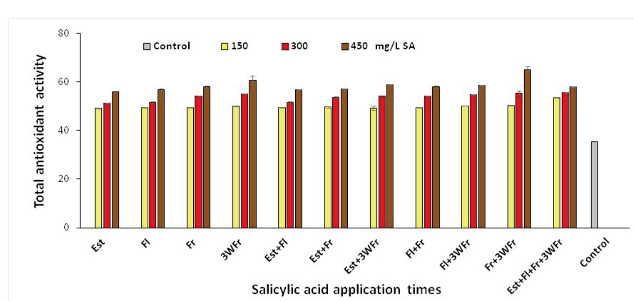

Fig. 2 - Effect of salicylic acid (SA) foliar application at different tomato plant phenological stages on fruit total antioxidant activity at greenhouse condition. Transplant establishment (Est), flowering (Fl), fruiting (Fr), three weeks after fruiting (3WFr).

(Shakirova, 2007). It is frequently hypothesized that SA has direct physiological effects on the activity of antioxidant enzymes which promote the synthesis of metabolites existing in fruits and vegetables, especially those with nutritional value in the product (Huang *et al.*, 2008 a).

Flavonoids

Flavonoids comprise a diverse group of natural compounds and are among the best-known natural phenols, exhibiting an array of chemical and biological pathways such as radical scavenging and antimicrobial activities. An increasing pattern in the amount of flavonoids was found by increasing SA concentration irrespective to application time. The highest amount of flavonoids was observed in 450 mg/L SA treatment when plants were treated either in three weeks after fruiting or fruiting plus three weeks after fruiting stages with about 1.18 times greater than control treatment (Table 1). Reports have been cited stating that exogenous SA applications boost the accumulation of flavonoids in several plant species (Tounekti *et al.*, 2013). On the other hand, research has proved that flavonoids possess antibiotic activities (Al-Matani *et al.*, 2015). This can generate debate as to whether higher amounts of flavonoids can contribute to a longer shelf life against the rot of perishable fruits like tomato. When vegetables are heated for special purposes in food industries, excess heat can cause degradations in flavonoids and thus can reduce its overall content (Sharma *et al.*, 2015).

Correlation between secondary metabolites and antioxidant activity

A Pearson correlation analysis was performed to determine relationships between the individual parameters phenolic compounds, flavonoids and ascorbic acid which contribute in antioxidant activity. Significant correlations were found for all measured traits with the highest effect of flavonoids on antioxidant activity; however, it was not a simple sum of their contribution (Table 2). This has been related to synergistic effect among all antioxidants and their interactions with other constituents of the fraction (Jimenez *et al.*, 2002; Lenucci *et al.*, 2006). Similar to our results, Ilahy *et al.* (2011) found a good significant correlation between antioxidant activity and main antioxidants (vitamin C, flavonoids and total phenols). Given the key role of SA in increasing ascorbic acid (Dat *et al.*, 1998), total phenolics and other secondary metabolites with antioxidant properties (Ranjbaran *et al.*, 2011), the rise in antioxidant activity can thus be explained.

Table 2 - Pearson correlation analysis between secondary metabolites and antioxidant activity

	Phenolic compounds	Flavonoid	Vitamin C
Antioxidant activity	0.621 (*)	0.823 (*)	0.639 (*)

(*) Significant differences at $p<0.05$.

4. Conclusions

Treatments with SA could be a promising tool to improve tomato yield, fruit quality attributes and health beneficial compounds (including phenolic compounds, vitamin C and flavonoids having antioxidant activity) because of its diverse regulatory roles in plant metabolism. The effect of exogenous SA on plant depends on the plant species, developmental stage, and the SA concentrations tested. Fruit setting stage and 3 weeks later are the best two important stages for SA application. A concentration of 300 mg/L SA for increased yield and 450 mg/L SA for improved fruit quality attributes are recommended. It is possible that exogenous application of SA, out of recommended rates, have negative or adverse effect on desired characteristics.

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