

# Influence of foliar-applied salicylic acid on growth, gas-exchange characteristics, and chlorophyll fluorescence in citrus under saline conditions

D. KHOSHBAKHT<sup>+</sup> and M.R. ASGHAREI

*Department of Horticultural Science, College of Agriculture, University of Urmia, West Azarbaijan, Iran*

## Abstract

Salicylic acid (SA) is a common, plant-produced signal molecule that is responsible for inducing tolerance to a number of biotic and abiotic stresses. Our experiment was therefore conducted to test whether the application of SA at various concentrations (0, 0.10, 0.50, and 1.00 mM) as a foliar spray would protect citrus seedlings (Valencia orange/Bakraii) subjected to salt stress (0, 25, 50, and 75 mM NaCl). Growth parameters, leaf chlorophyll (Chl) content, relative water content (RWC), maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ), and gas-exchange variables were negatively affected by salinity. In addition, leaf electrolyte leakage (EL) and proline content increased by salinity treatments. Application of SA increased net photosynthetic rate and proline content in salt stressed plants and may have contributed to the enhanced growth parameters. SA treated plants had greater Chl content and RWC compared with untreated plants when exposed to salt stress.  $F_v/F_m$  ratio and stomatal conductance were also significantly higher in SA treated plants under saline stress conditions. SA application reduced EL compared to untreated plants, indicating possible protection of integrity of the cellular membrane. It appeared that the best ameliorative remedies of SA were obtained when Valencia orange/Bakraii seedlings were sprayed by 0.50 and 1.00 mM solutions. Overall, the adverse effects of salt stress could be alleviated by exogenous application of SA.

*Additional key words:* abscisic acid; electrical conductivity; leaf area; photosystem II; physiological parameters.

## Introduction

The progressive salinization of agricultural land is considered as the major environmental factor limiting plant growth and productivity of the arid and semi-arid areas (Sudhir and Murthy 2004). In these areas, many soils and waters contain amounts of salts that can inhibit the growth and yield of citrus crops. Citrus is grown commercially in over 50 countries and ranks as the top fruit crop in world production (García-Sánchez *et al.* 2002). Citrus trees have been classified as the salt-sensitive crop (Storey and Walker 1999) as saline irrigation water reduces citrus tree growth and the fruit yield (Grieve *et al.* 2007). Salinity causes various injuries in plants, such as tissue burning, yield reduction, and finally plant death (Zekri and Parsons 1992), reduces water uptake and tree growth (Syvertsen

and Yelenosky 1988), causes leaf senescence, reduction of chlorophyll (Chl) content (Chen *et al.* 1991) and PSII activity (Nishihara *et al.* 2003), decreases stomatal conductance and net assimilation of CO<sub>2</sub> (García-Sánchez *et al.* 2002), increases membrane permeability (Dhindsa *et al.* 1981), and causes nutritional imbalances and toxicity (Grattan and Grieve 1992).

The development of methods inducing stress tolerance in plants is vital and still receives considerable attention. Approaches taken to develop stress-tolerant plants have included genetic engineering (McKersie *et al.* 1988), traditional breeding (Vettakkorumakankav *et al.* 1999), *in vitro* selection, and the use of growth regulators (Baninasab and Ghobadi 2011). Salicylic acid (SA) alters

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<sup>+</sup>Corresponding author; phone: +98 311 3913447, fax: +98 311 3913356, e-mail: [davod.khoshbakht@gmail.com](mailto:davod.khoshbakht@gmail.com)

**Abbreviations:** ABA – abscisic acid; ACC – 1-aminocyclopropane-1-carboxylic acid; C<sub>a</sub> – atmospheric CO<sub>2</sub> concentration; Chl – chlorophyll; DM – dry mass; EC – electrical conductivity; EL – electrolyte leakage; F<sub>0</sub> – minimal fluorescence yield at the dark-adapted state; FM – fresh mass; F<sub>m</sub> – maximal fluorescence yield at the dark-adapted state;  $F_v/F_m$  – maximum photochemical efficiency of PSII; g<sub>s</sub> – stomatal conductance; IAA – indole-3-acetic acid; LA – total leaf area; LN – number of leaves per plant; PC – proline content; P<sub>N</sub> – net photosynthetic rate; RH – relative humidity; ROS – reactive oxygen species; RWC – relative water content; S – salinity stress; SA – salicylic acid; TDM – total plant dry mass; TM – turgid mass; VOB – Valencia orange/Bakraii.

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key plant functions including nutrient uptake, membrane functioning (Glass and Dunlop 1974), water relations (Barkosky and Einhellig 1993), stomatal functioning (Aldesuquy *et al.* 1998), inhibition of ethylene biosynthesis (Leslie and Romani 1988, Srivastava and Dwivedi 2000), and increases growth (Rajasekaran and Blake 1999). The application of SA has resulted in tolerance of plants to many biotic and abiotic stresses including fungi, bacteria, viruses (Delaney *et al.* 1994), chilling (Senaratna *et al.*

2003), drought, and heat (Senaratna *et al.* 2000). Since SA is an endogenous growth regulator, it was important to study the influence of exogenous SA on growth processes in Valencia orange/Bakraii plants.

Therefore, the objectives of this work were to determine (1) the effect of salt stress and SA on the morphological and physiological changes in citrus plants; (2) whether a foliar supply of SA to Valencia orange/Bakraii might be a strategy for increasing its salt tolerance.

## Materials and methods

**Plant material, growth conditions, salinity, and SA treatments:** Eighteen-month-old grafted plant of Valencia orange (*Citrus sinensis*) scion on Iranian mandarin Bakraii (*Citrus reticulata* × *Citrus limetta*) was used in this experiment. Plants were grown under greenhouse conditions, located at Department of Horticultural Science, College of Agriculture, University of Urmia, Iran. This grafting combination of Valencia orange/Bakraii (scion/rootstock, VOB) was transplanted into 30 cm wide plastic pots containing fine sand. The temperature for day/night was 25–28/17°C, the relative humidity was 60–70%, and the average PAR was 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with 16-h photoperiod. Plants were irrigated in 2-d intervals with 0.5 L of water and fertilized with a commercial water soluble fertilizer containing macro and micro nutrients (*Floral Mixed fertilizer*, IFO, Italia). Afterwards, the seedlings of a similar size and appearance (the plant height was 140 cm) were sprayed two times (at 1-week interval) with 0 (control), 0.10, 0.50, and 1.00 mM SA until both sides of all the leaves were completely wet. Tween-20 at amount of 1% (v/v) was added to SA solution as a surfactant. Two days after the last foliar application of SA, all seedlings were exposed to salt treatments. Salt treatments (0, 25, 50, and 75 mM of NaCl, respectively) were added to the pots at 3-d intervals using 0.5 L of irrigation water. To avoid osmotic shock, the NaCl concentrations were increased gradually by adding increments of 25 mM NaCl every 3 d until the desired concentration was reached. Various analyses were performed 60 d after salt treatment.

**Growth characteristics:** At the end of the experiment, plants were harvested and the number of leaves was counted. Plants were washed with distilled water to remove adhering foreign particles. Leaf area was measured using a *Delta-T* leaf area meter (*Delta-T Devices Ltd.*, Cambridge, UK). The shoots and roots were dried separately at 80°C for 48 h and their dry masses were then recorded.

**Electrolyte leakage (EL)** was used to assess membrane permeability. EL was measured using an electrical conductivity meter (*CC-501*, *Elmetron*, Zabrze, Poland). Six leaf discs were taken from the youngest fully-expanded leaf on one randomly chosen plant per replicate sample (pot). After three washes with distilled water to remove surface contamination, the leaf discs were placed in test

tube containing 10 ml of distilled water. These samples were incubated for 24 h on a shaker at room temperature. The electrical conductivity (EC) of the solution (EC1) was read after the incubation. The same samples were then placed in an autoclave at 120°C for 20 min and the second EC reading (EC2) was taken after cooling the solution to room temperature. EL was then calculated as  $\text{EC1/EC2}$ , and expressed as a percentage (Lutts *et al.* 1995).

**Relative water content (RWC)** of leaves was determined following the method suggested by Barrs and Weatherley (1962). Ten leaf discs of 1 cm diameter were cut from the youngest fully-expanded leaf, using a leaf punch. Leaf discs were weighed (fresh mass, FM) and washed three times with double distilled water and placed into a 10 ml conical flask. Leaf discs were immersed in 10 ml distilled water for 4 h at 4°C in dark. Turgid mass (TM) of leaf discs was then measured and samples were dried in hot air oven at 70°C until constant dry mass (DM) was achieved. RWC was estimated using the following equation:  $\text{RWC [\%]} = (\text{FM} - \text{DM})/(\text{TM} - \text{DM}) \times 100$ .

**Total chlorophyll content** was determined according to Lichtenthaler (1987). After gas-exchange measurements, the total Chl was extracted from 500 mg of leaf discs that were chosen from the same leaf used for  $P_N$  measurements (avoiding major veins) using 80% aqueous acetone. Absorbance of centrifuged extracts was measured at 645 and 663 nm using a spectrophotometer (*U-2000*, *Hitachi Instruments*, Tokyo, Japan).

**Proline content (PC)** was determined according to the method described by Bates *et al.* (1973). Seedlings (0.5 g of fresh shoots) were homogenized in 10 ml of 3% (v/v) aqueous sulphosalicylic acid and filtered through a filter paper. Then, 2 ml of the filtrate was mixed with 2 ml of acid-ninhydrin reagent and 2 ml of glacial acetic acid in a test tube and the mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 ml of toluene, the chromophore-containing toluene fraction was aspirated, cooled to room temperature, and its absorbance measured at 520 nm using a spectrophotometer (*U-2000*, *Hitachi Instruments*, Tokyo, Japan). Appropriate PC standards (*Sigma Chemical Co.*, St. Louis, MO, USA) were included in order to calculate the concentration of

proline (PC, standard concentrations were between 0–100 ppm and absorbances were recorded at 520 nm) in each shoot sample.

**Maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ):** At the end of experiment, measurement of fluorescence parameters were performed between 9:00–11:00 h, using a *Plant Efficiency Analyzer (PEA, Hansatech Instrument Ltd., King's Lynn, Norfolk, UK)*. The undamaged mature leaves were darkened with a lightweight plastic leaf clips for 30 min before the measurement.  $F_v/F_m$  was calculated automatically as  $(F_m - F_0)/F_m$ ; where  $F_m$  and  $F_0$  were the maximum and basal fluorescence yields of dark-adapted leaves, respectively.

**Gas-exchange parameters:** The net photosynthetic rate ( $P_N$ ) and stomatal conductance ( $g_s$ ) were measured from 9:00–11:00 h at the end of the experiment using a portable photosynthetic system (*LI-6200, LI-COR Inc., Lincoln,*

NE, USA). A top, fully expanded leaf was clamped to the leaf chamber and the observations were recorded when RH and atmospheric  $CO_2$  concentration ( $C_a$ ) reached a stable value. PAR, air temperature, relative humidity, and  $CO_2$  concentration inside the sensor head were set at  $1,300 \pm 100 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ,  $31 \pm 2^\circ\text{C}$ , 60%, and  $335\text{--}340 \mu\text{mol m}^{-1}$ , respectively. The leaf was held in the chamber for 3 min to obtain stable reading.

**Statistical analysis:** The layout was as a  $4 \times 4$  factorial experiment in a complete randomized design, with five replications and three plants per replication (pot). Data were analyzed for significant differences using a factorial analysis of variance, with SA and NaCl concentrations as the main factors. Statistical analysis was performed using the *SAS* program version 9.1 (*SAS Inc., Cary, NC, USA*) and means were compared using the least significant differences (LSD) test at  $P < 0.05$ .

## Results

**Growth parameters:** A comparison of growth parameters between VOB seedlings grown in absence of salinity or in

the presence of 25, 50, and 75 mM NaCl and treated with SA is shown in Table 1. SA applied through foliar spray

Table 1. Leaf number (LN), leaf area (LA), total plant dry mass (TDM), leaf electrolyte leakage (EL), and relative water content (RWC) in Valencia orange/Iranian mandarin Bakraii seedlings treated with salicylic acid (SA) and NaCl. Means  $\pm$  SD of five independent measurements. Mean values followed by the same letters within a column are not significantly different at  $p < 0.05$ .

NaCl [mM]	SA [mM]	LN	LA [ $\text{cm}^2$ ]	TDM [g]	EL [%]	RWC [%]
0	0	$29 \pm 0.82^{\text{cd}}$	$29.42 \pm 0.82^{\text{c}}$	$21.90 \pm 0.82^{\text{c}}$	$14.5 \pm 1.63^{\text{hi}}$	$80.5 \pm 2.45^{\text{abc}}$
	0.1	$30 \pm 1.63^{\text{bc}}$	$31.16 \pm 0.80^{\text{b}}$	$24.00 \pm 0.88^{\text{b}}$	$14.2 \pm 1.54^{\text{hi}}$	$78.3 \pm 1.63^{\text{cd}}$
	0.5	$32 \pm 1.60^{\text{ab}}$	$33.52 \pm 0.84^{\text{a}}$	$26.40 \pm 0.84^{\text{a}}$	$13.5 \pm 2.45^{\text{i}}$	$82.4 \pm 1.63^{\text{ab}}$
	1	$34 \pm 1.53^{\text{a}}$	$32.64 \pm 0.81^{\text{a}}$	$27.60 \pm 1.63^{\text{a}}$	$13.4 \pm 1.68^{\text{i}}$	$83.5 \pm 2.25^{\text{a}}$
25	0	$23 \pm 1.63^{\text{fg}}$	$22.60 \pm 0.08^{\text{f}}$	$17.10 \pm 0.82^{\text{def}}$	$22.0 \pm 1.63^{\text{f}}$	$77.3 \pm 2.35^{\text{cd}}$
	0.1	$25 \pm 1.65^{\text{ef}}$	$23.00 \pm 0.01^{\text{f}}$	$17.50 \pm 0.87^{\text{de}}$	$19.0 \pm 0.82^{\text{g}}$	$77.4 \pm 2.44^{\text{cd}}$
	0.5	$27 \pm 2.45^{\text{de}}$	$25.27 \pm 1.63^{\text{e}}$	$18.90 \pm 0.86^{\text{d}}$	$15.6 \pm 1.66^{\text{hi}}$	$79.3 \pm 2.46^{\text{bc}}$
	1	$28 \pm 1.63^{\text{cd}}$	$26.90 \pm 0.82^{\text{d}}$	$18.00 \pm 1.63^{\text{de}}$	$16.2 \pm 1.63^{\text{h}}$	$77.5 \pm 2.43^{\text{cd}}$
50	0	$18 \pm 2.45^{\text{i}}$	$17.58 \pm 0.88^{\text{ij}}$	$13.70 \pm 0.88^{\text{h}}$	$39.0 \pm 0.82^{\text{b}}$	$69.1 \pm 1.82^{\text{f}}$
	0.1	$19 \pm 1.43^{\text{i}}$	$18.08 \pm 0.82^{\text{hi}}$	$14.30 \pm 0.82^{\text{h}}$	$34.0 \pm 1.63^{\text{c}}$	$73.2 \pm 2.44^{\text{e}}$
	0.5	$22 \pm 2.45^{\text{gh}}$	$20.86 \pm 0.85^{\text{g}}$	$16.20 \pm 1.63^{\text{efg}}$	$25.0 \pm 1.63^{\text{e}}$	$74.9 \pm 3.27^{\text{de}}$
	1	$24 \pm 2.48^{\text{fg}}$	$19.10 \pm 0.88^{\text{h}}$	$15.50 \pm 2.45^{\text{fgh}}$	$28.0 \pm 1.55^{\text{d}}$	$75.4 \pm 4.08^{\text{de}}$
75	0	$14 \pm 3.27^{\text{j}}$	$14.52 \pm 0.82^{\text{k}}$	$9.60 \pm 0.88^{\text{i}}$	$48.0 \pm 1.63^{\text{a}}$	$59.2 \pm 1.82^{\text{g}}$
	0.1	$15 \pm 2.44^{\text{j}}$	$14.73 \pm 0.82^{\text{k}}$	$10.80 \pm 1.63^{\text{i}}$	$45.7 \pm 2.87^{\text{a}}$	$66.3 \pm 3.27^{\text{f}}$
	0.5	$18 \pm 1.65^{\text{i}}$	$17.45 \pm 0.86^{\text{ij}}$	$13.70 \pm 1.68^{\text{h}}$	$35.0 \pm 1.67^{\text{c}}$	$68.2 \pm 1.63^{\text{f}}$
	1	$20 \pm 2.42^{\text{hi}}$	$16.87 \pm 0.83^{\text{j}}$	$14.40 \pm 1.58^{\text{gh}}$	$38.0 \pm 1.60^{\text{b}}$	$65.9 \pm 4.08^{\text{f}}$
Means for NaCl						
0		$31.20 \pm 2.38^{\text{a}}$	$31.70 \pm 1.76^{\text{a}}$	$24.90 \pm 2.47^{\text{a}}$	$13.9 \pm 1.74^{\text{d}}$	$81.2 \pm 2.76^{\text{a}}$
25		$25.70 \pm 2.59^{\text{b}}$	$24.40 \pm 1.98^{\text{b}}$	$17.90 \pm 1.19^{\text{b}}$	$18.2 \pm 2.94^{\text{c}}$	$77.9 \pm 2.35^{\text{b}}$
50		$20.70 \pm 3.19^{\text{c}}$	$18.90 \pm 1.49^{\text{c}}$	$14.90 \pm 1.74^{\text{c}}$	$31.5 \pm 5.74^{\text{b}}$	$73.1 \pm 3.65^{\text{c}}$
75		$16.70 \pm 3.34^{\text{d}}$	$15.80 \pm 1.41^{\text{d}}$	$12.10 \pm 2.44^{\text{d}}$	$41.7 \pm 5.81^{\text{a}}$	$64.9 \pm 4.30^{\text{d}}$
Means for SA						
	0	$21.00 \pm 6.13^{\text{c}}$	$21.00 \pm 5.86^{\text{c}}$	$15.50 \pm 4.72^{\text{c}}$	$30.8 \pm 13.79^{\text{a}}$	$71.5 \pm 8.67^{\text{c}}$
	0.1	$22.20 \pm 6.14^{\text{c}}$	$21.70 \pm 6.42^{\text{b}}$	$16.60 \pm 5.11^{\text{b}}$	$28.2 \pm 12.99^{\text{b}}$	$73.8 \pm 5.39^{\text{b}}$
	0.5	$24.70 \pm 5.74^{\text{b}}$	$24.10 \pm 6.40^{\text{a}}$	$18.80 \pm 5.05^{\text{a}}$	$22.2 \pm 8.96^{\text{d}}$	$76.2 \pm 5.89^{\text{a}}$
	1	$26.50 \pm 5.66^{\text{a}}$	$23.80 \pm 6.53^{\text{a}}$	$18.80 \pm 5.63^{\text{a}}$	$23.9 \pm 10.24^{\text{c}}$	$75.5 \pm 7.19^{\text{ab}}$

was effective within the range of 0.1–1.00 mM in protecting VOB seedlings against salt stress. TDM, LA, and LN were significantly affected by the salt stress and SA concentrations (Table 2). Salt stress significantly decreased TDM, LA, and LN in the VOB seedlings. The lowest LN (16.7), LA (15.8 cm<sup>2</sup>), and TDM (12.1 g) were observed in the seedlings treated with 75 mM NaCl, which were 46.4, 50.1, and 51.4% lower than those in the controls, respectively (Table 1). Exogenously applied SA resulted in significantly increased plant growth both under saline and non-saline conditions. The application of SA significantly increased TDM and LA compared with the control, with the largest increase in TDM, LA, and LN when using 0.50 or 1.00 mM SA (Table 1). In the absence of SA, the saline treatment (50 or 75 mM NaCl) diminished LA by about 50.6 and 40.2%, respectively. However, with the SA treatment (0.50 mM), the reduction of LA by salt stress was only about 20.1 and 18.6%, respectively. Application of SA significantly increased LN. The highest LN was obtained in seedlings treated with 1.00 mM SA (26.5), which was 26.2% higher than that in the control (Table 1). There was a significant interaction between salinity and SA concentration for TDM and LA (Table 2). However, at 0 and 25 mM NaCl, the greatest increase in LA occurred at 1.00 mM SA (33.5 and 26.9 cm<sup>2</sup>, respectively), while 0.50 mM SA was the most effective at 50 and 75 mM NaCl (20.8 and 17.4 cm<sup>2</sup>, respectively) (Table 1). Under 0 and 75 mM NaCl, the greatest increase in TDM occurred at 1.00 mM SA (27.6 and 14.4 g, respectively), while 0.50 mM SA was the most effective at 25 and 50 mM NaCl (18.9 and 16.2 g, respectively) (Table 1).

**Physiological and biochemical parameters:** Salt stress significantly increased EL, with maximum increase observed in plants grown with 75 mM NaCl (Tables 1, 2). The application of SA significantly decreased EL in leaf discs, with the largest decrease in EL measured when 0.50 mM SA was applied. There was a significant interaction between salinity and the SA concentration (Table 2). The SA treatment had no significant effect on EL in unstressed plants. Plants pretreated with 0.50 and 1.00 mM SA and subjected to 25 mM salt stress had similar EL rates in comparison with unstressed plants. When plants were subjected to salinity stress, leaf electrolyte leakage, a reflection of membrane damage, was reduced by 29.1, 35.9, and 27.1% in plants treated with 0.50 mM SA and subjected to 25, 50, and 75 mM NaCl, respectively.

RWC was significantly affected by both salinity and SA pretreatment (Table 2). Increasing the concentrations of NaCl from 0 to 75 mM lowered RWC in VOB plants (Table 1). All concentrations of SA increased RWC significantly compared with the control. However, 0.50 mM SA was the most effective (76.2%) (Table 1). There was a significant interaction between salinity and the SA concentration (Table 2). The SA treatment had no significant effect on RWC under 25 mM NaCl treatment. Plants

treated with SA maintained higher RWC when subjected to 50 and 75 mM NaCl treatments (Table 1).

The Chl content was affected by both salt stress and SA application (Table 2). Salinity stress decreased the Chl content dramatically in the leaves of salt-treated VOB seedlings. At 75 mM NaCl, the Chl content was minimal compared with those of the control and other salt concentrations (Table 3). Application of SA significantly increased Chl values compared with the control, with the largest increase when 1.00 mM SA was applied (Tables 2, 3). There was a significant interaction between salinity and SA concentrations (Table 2). Imposition of salt stress resulted in reduction in Chl under all treatments. However, the seedlings treated with SA exhibited lesser decline than the control seedlings (Table 3). There was a significant interaction between salinity and SA concentrations (Table 1). The highest Chl content in the leaves was observed with 1.00 mM SA at 0, 25, 50, and 75 mM NaCl [0.88, 0.78, 0.67, and 0.47 mg g<sup>-1</sup>(FM), respectively] (Table 3).

The PC content of VOB leaves was significantly influenced by the salt stress and SA application (Table 3). In the present study, the increase of PC content in the NaCl treated plants was noted, with the highest content being attained with 75 mM NaCl (Table 3). SA treatment increased the PC content in VOB leaves. The highest PC content was obtained from leaves of the seedlings treated with 1 mM SA [54.9 µmol g<sup>-1</sup>(FM)], which was 27.9% more than that of the control (Table 3). There was a significant interaction between salinity and SA concentrations (Table 2). Under 0, 25, and 75 mM NaCl, the greatest increase in the PC content occurred at 1.00 mM SA [39.3, 48.7, and 75.4 µmol g<sup>-1</sup>(FM), respectively], while 0.50 mM SA was the most effective at 50 mM NaCl [58.4 µmol g<sup>-1</sup>(FM)] (Table 3).

**Photosynthetic parameters:** The  $F_v/F_m$  was used as a noninvasive method to determine the functional state of the photosynthetic machinery. The  $F_v/F_m$  was significantly affected by salt stress and SA concentration (Table 2). Salinity stress decreased  $F_v/F_m$  in the leaves of the salt-treated VOB seedlings. The lowest  $F_v/F_m$  was observed in the leaves of seedlings treated with 75 mM NaCl (0.56), which was 30.9% lower than that in the control (Table 3). SA treatment significantly increased the  $F_v/F_m$  in VOB leaves. The highest  $F_v/F_m$  was observed in the leaves of seedlings treated with 0.50 and 1.00 mM SA (0.72) (Table 3). However, interaction between salinity and SA showed that  $F_v/F_m$  was not affected by SA under the nonsaline condition and 25 mM NaCl; under higher saline conditions, the maximum  $F_v/F_m$  in the leaves was observed with 1.00 mM SA (0.70 and 0.60 at 50 and 75 mM NaCl, respectively) (Table 3).

Salt stress significantly reduced the  $P_N$  and  $g_s$ , with maximum reduction observed in the plants grown with 75 mM NaCl (Tables 2, 3). Application of SA, especially at the highest concentration, significantly increased  $P_N$  and  $g_s$

Table 2. Linear correlations ( $r$  values) among the leaf number (LN), leaf area (LA), total plant dry mass (TDM), leaf electrolyte leakage (EL), relative water content (RWC), total chlorophyll concentration (Chl), proline content (PC), maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ), net photosynthetic rate ( $P_N$ ), and stomatal conductance ( $g_s$ ) of Valencia orange/Iranian mandarin Bakraii seedlings treated with salicylic acid (SA) and NaCl.  $|r| > 0.7$  most-significant correlation, \*\*\* –  $P < 0.001$ , \*\* –  $P < 0.01$ . Analysis of variance (ANOVA) for NaCl, SA concentration, and their interaction (NaCl  $\times$  SA). ns – not significant.

	LN	LA	TDM	EL	RWC	Chl	PC	$F_v/F_m$	$P_N$	$g_s$
Linear correlations										
LN	1	0.93***	0.93***	-0.89***	0.90***	0.91***	-0.65***	0.89***	0.92***	0.90***
LA		1	0.95***	-0.90***	0.85***	0.92***	-0.73***	0.90***	0.96***	0.95***
TDM			1	-0.84**	0.86***	0.88**	-0.68**	0.85***	0.93***	0.87**
EL				1	-0.87**	-0.96**	0.76***	-0.95***	-0.95***	-0.93***
RWC					1	0.91***	-0.70***	0.92***	0.96***	0.85***
Chl						1	-0.81***	0.97**	0.96***	0.95***
PC							1	-0.78***	-0.82***	-0.79**
$F_v/F_m$								1	0.94***	0.95***
$P_N$									1	0.85***
$g_s$										1
ANOVA, $p$ -values										
NaCl	0.0001	0.001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.001
SA	0.001	0.0001	0.001	0.0002	0.0001	0.0002	0.001	0.001	0.0001	0.0001
NaCl $\times$ SA	ns	0.0083	0.0351	0.0002	0.0221	0.0223	0.0255	0.01	0.032	0.0001

Table 3. Total chlorophyll concentration (Chl), proline content (PC), maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ), net photosynthetic rate ( $P_N$ ), and stomatal conductance ( $g_s$ ) in leaves of Valencia orange/Iranian mandarin Bakraii seedlings treated with salicylic acid (SA) and NaCl. Means  $\pm$  SD of five independent measurements. Mean values followed by the same letters within a column are not significantly different at  $p < 0.05$ .

NaCl [mM]	SA [mM]	Chl [mg g <sup>-1</sup> (DM)]	PC [μmol g <sup>-1</sup> (FM)]	$F_v/F_m$	$P_N$ [μmol(CO <sub>2</sub> ) m <sup>-2</sup> s <sup>-1</sup> ]	$g_s$ [mmol(H <sub>2</sub> O) m <sup>-2</sup> s <sup>-1</sup> ]
0	0	0.82 $\pm$ 0.02 <sup>b</sup>	27.80 $\pm$ 0.82 <sup>i</sup>	0.82 $\pm$ 0.02 <sup>a</sup>	7.80 $\pm$ 0.08 <sup>b</sup>	0.170 $\pm$ 0.01 <sup>a</sup>
	0.1	0.83 $\pm$ 0.03 <sup>b</sup>	29.10 $\pm$ 0.82 <sup>i</sup>	0.82 $\pm$ 0.02 <sup>a</sup>	8.40 $\pm$ 0.08 <sup>a</sup>	0.170 $\pm$ 0.01 <sup>a</sup>
	0.5	0.85 $\pm$ 0.02 <sup>ab</sup>	37.40 $\pm$ 0.82 <sup>g</sup>	0.81 $\pm$ 0.02 <sup>a</sup>	8.50 $\pm$ 0.08 <sup>a</sup>	0.160 $\pm$ 0.03 <sup>ab</sup>
	1	0.88 $\pm$ 0.01 <sup>a</sup>	39.30 $\pm$ 0.82 <sup>g</sup>	0.82 $\pm$ 0.02 <sup>a</sup>	8.50 $\pm$ 0.08 <sup>a</sup>	0.170 $\pm$ 0.02 <sup>a</sup>
25	0	0.76 $\pm$ 0.02 <sup>c</sup>	33.40 $\pm$ 0.82 <sup>h</sup>	0.78 $\pm$ 0.02 <sup>a</sup>	5.90 $\pm$ 0.08 <sup>e</sup>	0.120 $\pm$ 0.03 <sup>d</sup>
	0.1	0.75 $\pm$ 0.04 <sup>c</sup>	38.50 $\pm$ 0.82 <sup>g</sup>	0.78 $\pm$ 0.01 <sup>a</sup>	6.30 $\pm$ 0.02 <sup>d</sup>	0.140 $\pm$ 0.01 <sup>c</sup>
	0.5	0.77 $\pm$ 0.02 <sup>c</sup>	45.40 $\pm$ 4.08 <sup>ef</sup>	0.80 $\pm$ 0.02 <sup>a</sup>	6.70 $\pm$ 0.05 <sup>c</sup>	0.150 $\pm$ 0.01 <sup>bc</sup>
	1	0.78 $\pm$ 0.03 <sup>c</sup>	48.70 $\pm$ 1.63 <sup>e</sup>	0.79 $\pm$ 0.01 <sup>a</sup>	6.90 $\pm$ 0.08 <sup>c</sup>	0.160 $\pm$ 0.01 <sup>ab</sup>
50	0	0.52 $\pm$ 0.02 <sup>f</sup>	44.70 $\pm$ 3.27 <sup>f</sup>	0.61 $\pm$ 0.03 <sup>de</sup>	4.40 $\pm$ 0.08 <sup>g</sup>	0.088 $\pm$ 0.02 <sup>fg</sup>
	0.1	0.54 $\pm$ 0.02 <sup>f</sup>	45.40 $\pm$ 4.08 <sup>ef</sup>	0.64 $\pm$ 0.02 <sup>cd</sup>	4.70 $\pm$ 0.08 <sup>g</sup>	0.089 $\pm$ 0.01 <sup>fg</sup>
	0.5	0.62 $\pm$ 0.01 <sup>e</sup>	58.40 $\pm$ 1.63 <sup>d</sup>	0.68 $\pm$ 0.02 <sup>bc</sup>	5.40 $\pm$ 0.08 <sup>f</sup>	0.100 $\pm$ 0.03 <sup>e</sup>
	1	0.67 $\pm$ 0.02 <sup>d</sup>	56.30 $\pm$ 1.63 <sup>d</sup>	0.70 $\pm$ 0.02 <sup>b</sup>	5.10 $\pm$ 0.08 <sup>f</sup>	0.099 $\pm$ 0.01 <sup>ef</sup>
75	0	0.35 $\pm$ 0.02 <sup>h</sup>	65.70 $\pm$ 4.08 <sup>c</sup>	0.50 $\pm$ 0.04 <sup>g</sup>	2.40 $\pm$ 0.08 <sup>i</sup>	0.068 $\pm$ 0.02 <sup>i</sup>
	0.1	0.37 $\pm$ 0.03 <sup>h</sup>	70.80 $\pm$ 1.63 <sup>b</sup>	0.55 $\pm$ 0.04 <sup>f</sup>	2.80 $\pm$ 0.08 <sup>i</sup>	0.072 $\pm$ 0.01 <sup>hi</sup>
	0.5	0.45 $\pm$ 0.02 <sup>g</sup>	74.30 $\pm$ 3.27 <sup>ab</sup>	0.59 $\pm$ 0.01 <sup>ef</sup>	3.40 $\pm$ 0.01 <sup>h</sup>	0.079 $\pm$ 0.01 <sup>ghi</sup>
	1	0.47 $\pm$ 0.03 <sup>g</sup>	75.40 $\pm$ 4.08 <sup>a</sup>	0.60 $\pm$ 0.02 <sup>de</sup>	3.60 $\pm$ 0.08 <sup>h</sup>	0.083 $\pm$ 0.01 <sup>gh</sup>
Means for NaCl						
0		0.84 $\pm$ 0.03 <sup>a</sup>	33.40 $\pm$ 5.23 <sup>d</sup>	0.81 $\pm$ 0.02 <sup>a</sup>	8.30 $\pm$ 0.31 <sup>a</sup>	0.17 $\pm$ 0.01 <sup>a</sup>
25		0.76 $\pm$ 0.02 <sup>b</sup>	41.50 $\pm$ 6.47 <sup>c</sup>	0.78 $\pm$ 0.01 <sup>b</sup>	6.40 $\pm$ 0.49 <sup>b</sup>	0.14 $\pm$ 0.03 <sup>b</sup>
50		0.58 $\pm$ 0.06 <sup>c</sup>	51.50 $\pm$ 6.89 <sup>b</sup>	0.65 $\pm$ 0.04 <sup>c</sup>	4.90 $\pm$ 0.40 <sup>c</sup>	0.09 $\pm$ 0.02 <sup>c</sup>
75		0.41 $\pm$ 0.05 <sup>d</sup>	71.50 $\pm$ 4.96 <sup>a</sup>	0.56 $\pm$ 0.05 <sup>d</sup>	3.00 $\pm$ 0.49 <sup>d</sup>	0.07 $\pm$ 0.01 <sup>d</sup>
Means for SA						
0		0.61 $\pm$ 0.19 <sup>c</sup>	42.90 $\pm$ 15.17 <sup>c</sup>	0.67 $\pm$ 0.14 <sup>c</sup>	5.10 $\pm$ 2.05 <sup>c</sup>	0.114 $\pm$ 0.04 <sup>c</sup>
0.1		0.62 $\pm$ 0.19 <sup>c</sup>	45.90 $\pm$ 16.11 <sup>b</sup>	0.69 $\pm$ 0.11 <sup>b</sup>	5.50 $\pm$ 2.13 <sup>b</sup>	0.117 $\pm$ 0.04 <sup>bc</sup>
0.5		0.67 $\pm$ 0.16 <sup>b</sup>	53.80 $\pm$ 14.64 <sup>a</sup>	0.72 $\pm$ 0.09 <sup>a</sup>	6.00 $\pm$ 1.94 <sup>a</sup>	0.122 $\pm$ 0.03 <sup>ab</sup>
1		0.70 $\pm$ 0.16 <sup>a</sup>	54.90 $\pm$ 13.87 <sup>a</sup>	0.72 $\pm$ 0.16 <sup>a</sup>	6.00 $\pm$ 1.91 <sup>a</sup>	0.128 $\pm$ 0.04 <sup>a</sup>

compared with the controls (Table 3). SA treatment had no significant effect on  $g_s$  in the control plants. The plants treated with SA maintained higher  $P_N$  and  $g_s$  when subjected to salinity stress. Interaction between salinity and SA showed that exogenously applied SA at 0.50 and 1.00 mM caused a significant increase in  $P_N$  and  $g_s$  under the higher salt concentrations (50 and 75 mM NaCl) (Table 3).

**Correlations between physiological and morphological parameters:** In this study, the correlations between and among various physiological indices (*e.g.*, Chl, EL, RWC, PC,  $F_v/F_m$ ,  $P_N$ , and  $g_s$ ) and various morphological parameters (*e.g.*, LN, LA, and TDM) in VOB seedlings

subjected to salt stress were analyzed (Table 2). Results showed that significant correlations existed between and among these physiological indices and morphological parameters. These correlations suggested that TDM were positively correlated with LA, Chl, RWC,  $F_v/F_m$ ,  $P_N$ , and  $g_s$ , but negatively correlated with EL. Therefore, we concluded that SA ameliorated the negative effects of injury caused by salt stress through preventing decreases in Chl, RWC,  $F_v/F_m$ , and  $P_N$  and also by inhibiting increases in EL. Higher Chl and  $P_N$  values may have caused higher LN, LA, and TDM, since all the variables are closely related to photosynthetic capacity.

## Discussion

NaCl stress led to growth parameter reduction in VOB. Similar results were reported by numerous investigators (Zekri and Parsons 1992, García-Sánchez *et al.* 2000). Can *et al.* (2003) reported that irrigation of plants with saline water reduced the photosynthetic capacity per unit of area as well as further depressed vegetative growth of the whole tree. Besides, Cooper and Gorton (1952) mentioned that the decrement in the growth of the plants grown under salinity conditions might be attributed to an increase in the osmotic potential of the soil solution causing a marked depression in the water absorbing power of the plants. Our study demonstrated that the foliar application of SA prior to the exposure to salinity stress decreased the severity of the stress injury in the VOB plants. Coronado *et al.* (1998) reported that aqueous solutions of SA as a spray to shoots of soybean significantly increased the growth of shoots and roots under either greenhouse or field conditions. It was also reported that SA-treated maize plants showed higher dry mass as compared to those of untreated seedlings grown under salt stress (Gunes *et al.* 2007). This agrees with the findings of others that SA induces tolerance to many biotic (Delaney *et al.* 1994) and abiotic stresses (Senaratna *et al.* 2000). An increase in growth parameters of salt affected plants in response to SA might be related to the protective role of SA on membranes that might increase the tolerance of plants to salt stress (Aftab *et al.* 2010). The promoting effect of SA on the leaf area was attributed to its important role in activating cell division. In addition, Raskin (1992) mentioned that enhancing effect of SA on the availability and movement of nutrients could result in stimulating different nutrients in the leaves. Foliar application of SA increased the leaf area of sugarcane (Zhou *et al.* 1999). The exogenous application of SA prevented the lowering of indolyl acetic acid and cytokinin contents in salinity stressed wheat plants resulting in the cell division in root apical meristem, thereby increasing growth and productivity of plants (Shakirova *et al.* 2003). The exogenous application of SA resulted in the accumulation of abscisic acid (ABA) which might have contributed to the preadaptation of seedlings to salinity stress as ABA induces the synthesis of a wide

range of antistress proteins, thereby providing protection to the plants (Shakirova *et al.* 2003).

An increase in electrical conductivity indicates elevated leakiness of ions due to a loss of membrane integrity. This is an inherent feature of plants which are exposed to stresses such as salinity (Sharma *et al.* 2011). This study showed that SA reduced the ion leakage (measured as electrolytes) in salt stressed VOB plants indicating that SA treatment has facilitated the maintenance of membrane functions under stress conditions. Maintaining integrity of cellular membranes under salt stress is considered an integral part of salinity tolerance mechanism (Stevens *et al.* 2006). In agreement with our results, strawberry plants treated with SA showed far lesser electrolyte leakage than control plants after salt stress (Yildirim *et al.* 2008). SA causes increase in the activities of antioxidant enzymes which, in turn, protect plants against the generation of ROS and membrane injury, or may result in the synthesis of other substances, which have a protective effect on plants growing under salt stress (Xu *et al.* 2008).

The RWC is a useful measure of the physiological water status of plants. RWC in leaves is known as an alternative measure of plant water status, reflecting the metabolic activity in tissues (González and González-Vilar 2001). RWC was reduced by salinity that was in agreement with Stepien and Klobus (2006). RWC reduction indicated a loss of turgor that resulted in limited water availability for the cell extension process (Katerji *et al.* 1997) that may result from lower water availability under stress conditions (Shalhevet 1993), and/or root systems which are not able to compensate for water lost by transpiration through a reduction of the absorbing surface (Gadallah 2000). SA is reported to increase RWC and water potential tolerance of plants to salt stress (Tari *et al.* 2002). This is probably due to the fact that SA increases ABA, which ultimately helps in maintaining better water balance in the plants (Shakirova *et al.* 2003a). SA acts as a moderate stress, having an effect on the oxidative status of the plant similar to that of stress acclimating processes. A rapid transient increase in ROS is followed by enhanced antioxidative capacity, which protects the plant from the severe damage

caused by subsequent abiotic stress factors (Horvath *et al.* 2007).

The PC content of VOB plants under salt stress was higher than that of the control and SA increased this variable. PC has multiple functions, such as osmotic pressure regulation, protection of membrane integrity, stabilization of enzymes/proteins, maintaining appropriate NADP<sup>+</sup>/NADPH ratios, and scavenging free radicals (Hare and Cress 1997). Accumulation of PC under stress conditions, such as high salinity, has been correlated with stress tolerance (Misra and Gupta 2005). We observed a significant increase in the PC content after both treatments (SA and NaCl), which might be attributed to the strategies adapted by plants to cope with stress conditions. Since PC is an important component of the adaptation of plants to salinity, pretreatment with SA contributes to the accumulation of this amino acid under stress through maintaining the enhanced concentration of ABA in the seedlings (Sakhabutdinova *et al.* 2003).

In the present investigation, Chl,  $P_N$ ,  $g_s$ , and  $F_v/F_m$  decreased with increasing salinity. A decrease in the leaf Chl content has been described in citrus rootstocks irrigated with high NaCl concentration (García-Sánchez *et al.* 2002). This loss of Chl could be associated with accumulation of Cl<sup>-</sup> and Na<sup>+</sup> in the leaves (Anjum 2007). The higher Chl values of SA-treated VOB leaves may be related to the influence of SA on endogenous cytokinin contents. SA-treated plants synthesized more cytokinins (Shakirova *et al.* 2003a) which, in turn, enhanced chloroplast differentiation, Chl biosynthesis, and prevented Chl degradation (Fletcher *et al.* 1982). Downes and Crowell (1998) established that SA inhibited the activity of ACC (1-aminocyclopropane-1-carboxylic acid) synthase enzyme, preventing the formation of ethylene and Chl alteration.

The  $F_v/F_m$  is correlated with the efficiency of leaf photosynthesis. A decline in this ratio provides an indicator of photoinhibitory damage caused by the incident photon flux density when plants are subjected to a wide range of environmental stresses (Björkman and Demming 1987). Maintenance of the  $F_v/F_m$  in SA-treated plants under salt stress has been observed in previous studies (Szepesi *et al.* 2005).  $P_N$  showed a highly significant correlation with  $g_s$  which indicated that stomatal closure drove, at least in part, photosynthetic rate reduction under salt stress. Earlier studies have indicated that salt stress reduced  $P_N$  and  $g_s$  in the leaves of many crops (Poór *et al.* 2011, Syeed *et al.* 2011). A decline in photosynthetic capacity under salt stress is often associated with the generation of ROS (Noreen *et al.* 2010). Salt stress hinders photosynthesis at multiple levels, such as pigments, stomatal functioning, gas exchange, structure and function

of thylakoid membrane, electron transport, and enzyme activities, by hampering the oxidative stress mitigation mechanisms and cellular metabolism of plants (Sudhir and Murthy 2004). Excessive salt concentrations might cause the closure of stomata, thereby decreasing the partial CO<sub>2</sub> pressure and internal CO<sub>2</sub> concentration and consequently resulting in a decreased  $P_N$  (Bethke and Drew 1992). The SA-enhanced  $P_N$  under salt stress found in our study is in agreement with that of some earlier studies on mustard and *Artemisia annua* (Aftab *et al.* 2011, Syeed *et al.* 2011). Shi *et al.* (2006) showed that SA application improved net photosynthesis; this could be attributed to the role of SA in improving the functional state of the photosynthetic machinery in plants either by the mobilization of internal tissue nitrate or by Chl biosynthesis. Furthermore, SA has also been reported to enhance photosynthetic capacity in maize through stimulation of Rubisco activity (Khodary 2004). Khan *et al.* (2003) reported an increase in transpiration rate and  $g_s$  in response to foliar application of SA and other salicylates in corn and soybean. In another study carried out in soybean, foliar application of SA enhanced the WUE,  $E$ , and  $C_i$ . This positive effect of SA could be attributed to an increased CO<sub>2</sub> assimilation and photosynthetic rate and increased mineral uptake by the stressed plant under SA treatment (Khan *et al.* 2003). In soybean plants, treated with SA, pigment contents as well as the rate of photosynthesis increased (Zhao *et al.* 1995). This enhanced photosynthetic activity increased sap production in the leaf lamella which resulted in maintenance of relative water content in leaves and in better growth (Hayat *et al.* 2010). Foliar application of SA is also involved in stomatal regulation thereby controlling photosynthetic rate (Khan *et al.* 2003). Moreover, SA acts as one of antioxidant substances concentrated in the chloroplast and protects the photosynthetic apparatus when a plant is subjected to stress, by scavenging the excessively ROS known as free radicals. Such effects might be due to protecting the endogenous antioxidant systems often correlated with increased resistance to oxidative stress and/or controlling the amount of free radicals within plant tissues (Khan *et al.* 2003).

**Conclusion:** In summary, this study revealed that salt stress had inhibitory effects on the vegetative growth of Valencia orange/Bakraii seedlings. SA treatments could ameliorate the negative effect of salinity on the growth of Valencia orange/Bakraii and SA could be used as a potential growth regulator to improve plant salinity tolerance. SA applied at 0.50 and 1.00 mM were the most effective concentrations in providing Valencia orange/Bakraii seedlings with salt tolerance, especially at higher concentrations of NaCl.

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