Response of Citrus to Exogenously Applied Salicylate Compounds during Abiotic and Biotic Stress

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Citrus is exposed to a number of abiotic and biotic stresses that limit its productivity. Exogenous application of salicylic acid (SA) can induce plant defense mechanisms against environmental stresses. We investigated the effect of exogenously applied SA in alleviating stress damage in citrus during heat, cold, and disease stresses. Sodium salicylate (Na-SA) reduced the electrolyte leakage percentage (ELP) in both heat (up to 84%) and cold (up to 20%) stressed plants and maintained cell integrity of citrus leaves. However, the protective effect of Na-SA was concentration dependent with lower (<0.08 mM) and higher (>0.20 mM) concentrations failing to induce heat or cold tolerance. A narrow concentration range of 0.10 to 0.16 mM was most effective in protecting citrus from heat and cold stresses. Application of 0.14 to 0.18 mM Na-SA to Huanglongbing (HLB) -infected citrus trees increased plant pH from 6.1 to 6.5 compared to the untreated control. Salicylic acid applied to HLB-infected citrus trees also induced new foliage growth and flowering. Collectively, our results suggest that SA applied at appropriate concentrations can alleviate heat, cold, and disease stresses in citrus. In addition, appropriate concentrations of SA can be used to regulate the young foliage emergence and flowering in HLB infected citrus trees to compensate for the severe defoliation and regulate plant metabolic processes. Abbreviations: ELP Electrolyte leakage percentage; HLB Huanglongbing; K-SA Potassium Salicylate; Na-SA Sodium Salicylate; SA Salicylic acid.

Citrus is an economically important crop of Florida and the United States. However, citrus is exposed to a number of abiotic (temperature, drought, or salinity) or biotic (insect, pest and disease) stresses that limit its yield potential. Citrus can be grown in a variety of arid and humid climates, and can withstand temperatures ranging from –2.2 to 40.6 °C, but it performs best at temperatures between 15.6 and 30 °C (Parsons and Beck, 2004). Citrus trees are very sensitive to cold stress and are often damaged seriously by chilling episodes frequently occurring in winters. Freezing temperatures can cause extracellular ice formation, which lowers apoplastic water potential, dehydrates the symplast, and destabilizes cellular membranes (Steponkus, 1984), thus decreasing citrus productivity and profitability.

Citrus is also susceptible to a large number of diseases and insect attacks. Florida’s citrus industry is being ravaged by a devastating disease, huanglongbing (HLB). Huanglongbing is presumed to be caused by Candidatus Liberibacter asiaticus (Las), a phloem-limited gram-negative fastidious bacterium that affects the cytoplasmic continuity between adjacent sieve tube elements and sieve pores (Bove, 2006). The phloem plugging and disruption of transport of carbohydrates and related metabolites triggers various physiological changes in the tree. Reduced source-sink-transfer of phloem sap leads to starch accumulation and yellow shoot syndromes of citrus leaves, disorganized proliferation of phloem in the leaf veins, fewer new flushed of foliage, and atrophy of flower buds. The symptoms further lead to root starvation, reduced nutrient uptake and water conductance, and shoot dieback (Aubert, 2008). The fruit of diseased trees are misshapen, smaller in size, and do not color properly (Bove, 2006). Consequently, citrus trees infected with HLB may die within a few years and never produce usable fruit.

It is critical to protect citrus from the damage caused by abiotic and biotic stresses. Both abiotic and biotic stresses increase production of free radicals such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (Foyer et al., 1997), which are responsible for oxidative damage to membrane lipids and membrane peroxidation (Larkindale and Knight, 2002). Therefore, prevention of oxidative damage to cells during stress has been suggested as one of the mechanisms of stress tolerance (He et al., 2002). These mechanisms can also be induced or enhanced by the exogenous application of salicylic acid (SA) (Larkindale and Knight, 2002).

Salicylic acid is a natural signal molecule, which plays an important role in regulating a number of physiological processes in plants (Raskin, 1992). These processes may include flowering, new foliage growth, thermogenesis, and responses to biotic and abiotic stresses. Exogenously applied SA has resulted in tolerance of plants to many abiotic stresses including heat (Dat et al. 1998a and 1998b), cold (Senaratna et al. 2000), drought (Senaratna et al. 2000), and salinity (Shi et al., 2006). Salicylic acid is integral to the establishment of systemic acquired resistance (SAR) against pathogen attack (Heil and Bostock, 2002). The importance of SA in the induction of defense responses against pathogen attack is well documented in various crops such as tobacco, cucumber, common beans, and Arabidopsis (Chen et al., 1999; Clarke et al., 1998; Faheed and Mahmoud, 2006; Raskin 1992). However, the effect of SA in protecting plants from environmental stresses is concentration dependent. Salicylic acid exhibited a protective effect against heat stress in mustard seedlings only in a narrow

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concentration range (0.01–0.1 mM) and had no protective effect at lower (0.001 mM) or higher (1 mM) concentrations (Dat et al., 1998b). Salicylic acid concentrations of 1 mM have been found to have adverse effects on the growth of bean and tomato plants (Senaratna, et al. 2000). Therefore, determining the optimum concentration of SA is crucial for improving stress tolerance in plants.

Several approaches have been taken to assess stress tolerance in plants including assessments of membrane integrity (Heckman et al., 2002), anti-oxidant metabolism (He et al., 2002), \( \text{H}_2\text{O}_2 \) production, and SA genetic pathways (Snyman and Cronje, 2008). Maintaining integrity of cellular membranes under stress conditions is considered an integral part of tolerance mechanisms. Electrolyte leakage percentage (ELP), which represents cell membrane injury, has been correlated with extent of damage due to various stress injuries (Heckman et al. 2002; Shi et al., 2006). Therefore, it can be successfully used as an experimental screen for stress-tolerance to evaluate various stress injuries (Heckman et al., 2002; Shi et al., 2006). Besides ELP, plant pH and total dissolved solids or °Brix can also be used as indicators of plant health. Plant pH relates to the acid/base balance and has been used to partition the healthy and diseased plants of various crops such as wheat, corn, sunflower, and tobacco for which the diseased plants were more acidic than the healthy plants (Harvey, 1920; Hurd-Karrer, 1925; Noyes and Hancock, 1981). Leaf brix, which is an indicator of balance of nutrients and photosyneths, can be used as a measure of plant health.

In our present work, we investigated the role of exogenously applied salicylate compounds for enhancing abiotic stress tolerance and improving health of HLB infected citrus trees. Specifically, the objectives of this study were to 1) investigate whether exogenously applied salicylate will reduce cell electrolyte leakage of heat and cold stressed citrus leaves as a mechanism of stress tolerance; 2) determine the optimum salicylate concentration for alleviating stress damage in citrus; and 3) evaluate the effect of salicylate on disease stress, flowering, and leaf flush of HLB-infected citrus trees.

### Materials and Methods

**Plant material and sample preparation.**

**Laboratory experiment.** For heat and cold stress treatments, 4- to 5-month-old leaves were collected from ‘Valencia’ orange plants [Citrus sinensis (L.) Osb.] grown in the greenhouse facilities of the Citrus Research and Education Center (CREC), Lake Alfred, FL. For studying the effect of sodium salicylate (Na-SA) on HLB-infected citrus trees, HLB symptomatic and PCR positive leaves were collected from mature HLB-infected and healthy ‘Hamlin’ orange trees [C. sinensis (L.) Osb.] on Swingle citrumelo [C. paradisi Macf. × Poncirus trifoliata (L.) Raf.] rootstock growing in a grove at CREC. Leaves were cut into three leaf segments of approximately 15 cm² area, washed with deionized (DI) water, dried, and placed on filter papers moistened with test solutions in glass petri dishes with 9-cm diameter. All laboratory experiments consisted of randomized complete-block design (RCBD) with three replications. The exact number of experimental units varied with the concentrations used and the number is described with each specific experiment separately.

**Field experiment.** To evaluate the effect of exogenously applied salicylate in protecting citrus trees from heat and cold stresses under field conditions, a commercial citrus grove (Auburndale, FL) planted with 2-year-old ‘Hamlin’ orange trees on Swingle citrumelo was used. The trees were irrigated and fertilized with microsprinkler fertigation daily using a balanced mineral nutrition of 13 essential nutrient elements (N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, B, Mo, Cl). The experiment was conducted during Nov.–Dec. 2010 and utilized a RCBD with eight treatments and four replications (total trees = 32). The treatments were either foliar spray or soil drench application of Na-SA at 0, 0.10, 0.15, or 0.20 mM concentrations in water. For foliar treatment, each tree was sprayed using a hand sprayer to apply 2 L per tree of salicylate solution specific for each concentration. For soil drench application, 4 L of salicylate solution was applied to the root zone of each tree. Eight leaf samples (4–5 months old) were collected from each tree at 1, 7, 14, 21, and 28 d after salicylate application. Thereafter, leaves were exposed to heat or cold stress and ELP was determined for leaf samples collected at different time intervals. Mean temperature during the experiment ranged from 8 to 22 °C. The average temperatures post foliar or drench applications were 22, 20, 21, 11, and 8 °C for sampling intervals of 1, 7, 14, 21, and 28 d, respectively.

To evaluate the effect of Na-SA on PCR positive HLB symptomatic trees, a HLB-infected commercial citrus grove (Garret grove, Haines City, FL) was used. The experiment consisted of a RCBD with three replications. The treatments consisted of five concentrations of Na-SA (0, 0.10, 0.14, 0.18, and 0.22 mM) with or without the addition of Harrell’s Max Ornamental 9–3–6 [N–P₂O₅–K₂O] nutrient solution (Harrell’s Professional Fertilizer Solutions, Lakeland, FL) thus making 10 treatments (5 Na-SA concentrations × 2 nutrient solution treatments) and total 30 trees. The trees were sprayed using a hand sprayer to apply 1.5 L of solution to each tree. Harrell’s nutrient solution was derived from calcium nitrate, potassium nitrate, phosphoric acid, magnesium nitrate, U-32, copper-EDTA, iron-EDTA, Mn-EDTA, Zn-EDTA, sodium borate, sodium molybdate and nickel nitrate. Four to 6 month old HLB symptomatic leaves were collected every 3rd to 4th day for 14 d. The collected leaf samples were used to analyze ELP, brix, and pH.

### Heat stress

**Laboratory experiment.** Leaf segments placed in petri dishes were uniformly sprayed with 3 mL Na-SA or potassium salicylate (K-SA) at various concentrations (0, 0.10, 0.20, 0.30, and 0.40 mM) using a spray bottle. The concentration range was selected based on the concentrations (0.1–0.5 mM SA) reported to prevent heat stress damage in various crops including mustard, tomato, and beans (Dat et al., 1998b; Senaratna et al., 2000). After 3 h of Na-SA treatment, the leaves were exposed to heat stress at 50 °C for 5 h in the dark. Leaves in petri dishes were kept at room temperature for 16 h of recovery period after which electrolyte leakage percentage (ELP) was determined. Our preliminary experiments showed that Na-SA significantly reduced ELP at the concentration range of 0.1 to 0.2 mM and K-SA significantly reduced ELP at the concentration range of 0.2 to 0.4 mM. Therefore, the following experiments were conducted only for the effective concentration range of Na-SA (0, 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 mM) and K-SA (0, 0.225, 0.250, 0.275, 0.300, 0.325, 0.350, and 0.400 mM). Therefore, the total petri dishes for the Na-SA experiment were 21 (7 × 3) and for the K-SA experiment were 24 (8 × 3).

**Field experiment.** Leaves collected at different times from the trees pretreated with Na-SA were subjected to heat stress (50 °C for 5 h). Thereafter, leaves were given a 16-h recovery period and ELP was determined. Heat stress determination procedures were identical to those described earlier.

**Measurement of ELP for heat stressed leaves.** Leaf seg-
ments were washed with DI water and five leaf discs with 10-mm diameter were cut from each leaf segment. Leaf discs were rinsed three times with DI water. Electrolyte leakage was used to assess membrane permeability using the method described by Sairam et al. (1997). Leaf discs were placed into glass vials containing 15 ml DI water and covered with lids. Glass vials were placed in a forced draft oven at 40 °C for 30 min. Thereafter, samples were stirred and electrical conductivity (EC1) was measured after the samples attained room temperature using a portable conductivity meter (Thermo Fisher Scientific Inc, Waltham, MA). Then the glass vials were heated in a temperature-controlled water bath at 95 °C for 20 min and electrical conductivity (EC2) was measured. The EC2 was measured for the samples at room temperature. Electrolyte leakage was calculated as the ratio of EC1 to EC2 and expressed as percent.

Cold stress

**Laboratory experiment.** Leaf segments in the petri dishes were sprayed uniformly with 0, 0.08, 0.12, 0.16, 0.20, or 0.24 mM Na-SA using a hand atomizer, thus making a total of 18 petri dishes (6 concentrations × 3 replications). Leaves were exposed to cold stress for 24 h after the Na-SA treatment. For cold stress treatment, five leaf discs of 6.7-mm diameter were cut and placed into glass test tubes. Test tubes were then exposed to a series of freezing temperatures as described by Wiltbank and Oswalt (1983). Tubes were placed into seven test tube racks specific for each freezing temperature (−2.2, −3.3, −4.4, −5.6, −6.7, and −7.8 °C) and room temperature (25 °C). Six test tube racks for freezing treatment were placed into an ethylene glycol cold bath (Forma Scientific, Marietta, OH) precooled to −2.2 °C. A small amount of ice was added to each test tube to prevent the leaf discs from freezing. The rack for −2.2 °C was removed after 1 h and the bath temperature was lowered to the next freezing temperature (−3.3 °C in this case). This process was repeated until the last rack of −7.8 °C was removed from the freeze bath. Once the test tubes reached room temperature, 10 mL DI water was added to each test tube, covered with the Para film and kept overnight at room temperature to determine ELP.

**Field experiment.** Eight leaf samples were collected from each tree (trees = 32 as described earlier under field experiment description) at 1, 7, 14, 21, and 28 d after Na-SA treatment. Leaves collected at different times were subjected to cold stress as described for the laboratory experiment.

**Measurement of electrolyte leakage for cold stressed leaves.** After the cold stress treatment, the solution in each test tube was stirred and electrical conductivity (EC1) was measured. All test tube racks were covered with aluminum foil and autoclaved at 120 °C and 20 psi for 20 min using a steam sterilizer (Consolidated Stills and Sterilizers Boston, MA). The samples were kept overnight at room temperature and electrical conductivity (EC2) was measured following day after stirring the samples. Electrolyte leakage was calculated as the ratio of EC1 to EC2 and expressed as percent.

Disease stress

**Laboratory experiment.** Leaf segments in Petri dishes were sprayed uniformly with 9 concentrations of Na-SA (0, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, and 0.22 mM). The experiment had three replications making a total of 27 petri dishes. After 24 h, the leaves were washed with DI water and leaf discs were cut to determine ELP, pH, and brix. Electrolyte leakage percentage was determined using the methods described earlier. Thereafter, brix of the same solution was measured using a Digital Palette Portable Brix/Sucrose Refractometer (National Microscope Exchange, Carnation, WA). Solution pH was measured using a Corning 450 (Corning, Inc., Corning, NY) pH meter.

**Field experiment.** The 30 trees mentioned earlier under field experiment description were used to measure ELP, Brix, and sap pH using the methods described for the laboratory experiment. The morphological observations of young foliage growth were scored on a scale of 0 to 10 and for flowering on a scale of 0 to 5.

Statistical analysis

All data were analyzed using SAS statistical software (SAS Institute, Inc., 2003). Percentage data were arcsin transformed to validate the analysis of variance (ANOVA) assumption of normality and then subjected to statistical analysis. Original data are presented in the tables and figures. ANOVA was performed and means were separated using Fisher’s protected least significant difference (LSD) at 5% confidence interval. To determine the freezing points of citrus trees treated with different Na-SA concentrations in the cold stress experiment, average ELP for each treatment was plotted against temperature and citrus freezing point was defined as the temperature (LT50) corresponding to the 50% electrolyte leakage.

Results

Tolerance to heat stress

**Laboratory experiment.** Heat-stressed citrus leaves had significantly higher ELP than unstressed leaves (control). However, Na-SA decreased the ELP and cell membrane damage caused by heat stress. Electrolyte leakage decreased by 50% and 35% with 0.12 and 0.16 mM concentrations of Na-SA, respectively (Fig 1A). Very low (0.04 and 0.08 mM) and high (0.24 mM) Na-SA concentrations did not improve the tolerance of citrus leaves to heat stress. The regression analysis of Na-SA concentrations for ELP showed that 0.14 mM was the appropriate concentration for protecting citrus from heat-related cell membrane damage (Fig. 1B).

On the other hand, K-SA was effective at higher concentrations than Na-SA. The ELP decreased by 53% and 27% at 0.25 and 0.30 mM K-SA, respectively (Fig. 2A). The lower (0.20 and 0.225 mM) and higher (>0.30 mM) K-SA concentrations did not decrease ELP in the heat-stressed leaves. The regression analysis of K-SA concentrations for ELP showed that 0.29 mM was the optimum concentration that prevented heat stress (Fig. 2B).

**Field experiment.** Evaluation of heat stress tolerance in field conditions showed that the damaging effects of high temperature on cell membranes were reduced with Na-SA application (Fig. 3). After 1 d following SA spray, all Na-SA concentrations reduced the ELP. However, after 7 d only 0.10 mM Na-SA decreased ELP from heat stressed leaves. On the other hand 0.15 and 0.20 mM Na-SA treatments had higher ELP than control treatments. When the heat stress was induced after 14 d following Na-SA spray, 0.10 mM was also unable to prevent the leakage of cell contents from heat-stressed citrus leaves.

Tolerance to cold stress

**Laboratory experiment.** Sodium salicylate reduced the ELP of cold-stressed citrus leaves (Fig. 4A). Although all concentrations of Na-SA lowered the ELP, maximum ELP reduction (20%) was observed with 0.16 mM Na-SA. Regression analysis for ELP indicated that 0.14 mM was the appropriate concentration (R² =
0.90*** to prevent citrus leaves from cold stress. The curve of ELP at different temperatures for 0 and 0.16 mM Na-SA treatments showed that SA reduced ELP at all freezing temperatures (Fig. 4B). Freezing points or the temperatures at which 50% of the electrolytes leaked from leaf cells were –4.2 °C for 0 mM Na-SA and –6.7 °C for 0.16 mM Na-SA treatment, thus SA application lowered the freezing point of citrus by 2.5 °C.

**FIELD EXPERIMENT.** Sodium salicylate-treated cold-stressed citrus leaves had lower ELP than untreated leaves (Fig. 5A). The reduction in ELP was up to 15% when the leaves were subjected to cold stress 1 d after the Na-SA spray and decreased to 12% after 7 d. At 7 d following Na-SA spray 0.10 and 0.15 mM Na-SA were more effective in preventing cell membrane leakage than 0.20 mM Na-SA. However, after 14 and 21 d all three concentrations of Na-SA lowered the ELP. Sodium salicylate application also lowered the freezing points of citrus trees (Fig 5B). Three concentrations performed equally when leaves were subjected to cold stress 1 d after the spray (lowered freezing points from –6.7 °C to approximately –8 °C). Among Na-SA treatments, 0.15 mM performed better (lowered freezing points from –3.5 °C to approximately –5 °C) than the other concentrations. Overall the decrease in freezing points was up to 1.4 °C at different time periods.

Electrolyte leakage for experimental treatments at each freezing temperature varied when leaves were subjected to cold stress at different times (Table 1). When the leaves were subjected to cold stress 1 d after Na-SA spray, ELP decreased at –6.7 °C (20% reduction) and –4.4 °C (35% reduction). At 7 d, the Na-SA

![Fig. 1 Electrolyte leakage of citrus leaves (A) not subjected to heat stress (Control) or subjected to heat stress after spraying with various concentrations of Na-SA and (B) regression curve to determine appropriate concentration. Values are means, vertical bars represent SE of means (n=3).](image)

![Fig. 2 Electrolyte leakage of citrus leaves (A) not subjected to heat stress (Control) or subjected to heat stress after spraying with various concentrations of K-SA and (B) regression curve to determine appropriate concentration. Values are means, vertical bars represent SE of means (n=3).](image)
reduced ELP from 15% to 25% at –6.7, –5.6, and –4.4 °C, when maximum reduction in ELP was in 0.15 mM Na-SA treatment. Sodium salicylate treated citrus trees had lower ELP than the untreated trees at –7.8, –5.6, and –4.4 °C after 14 d of Na-SA application. The ELP was lowered only at –7.8 °C and –6.7 °C after 21 d and at –7.8 °C after 28 d following Na-SA spray. However at 21 and 28 d, all Na-SA concentrations were equally effective in protecting citrus leaves from the cell membrane damage caused by cold stress.

Tolerance to disease stress

LABORATORY EXPERIMENT. Sodium salicylate affected both ELP and pH of HLB-infected citrus leaves. Electrolyte leakage was lower in the healthy control leaves (11%) than the untreated, HLB-infected leaves (15%) (Fig.6A). Application of Na-SA reduced the ELP of HLB-infected leaves at 0.14 mM (32%) and 0.16 mM (22%) concentrations. Electrolyte leakage at these Na-SA concentrations was equal to the healthy leaves. Healthy citrus leaves had higher pH (6.6) than HLB-infected, untreated leaves for which the pH was 6.1 (Fig. 6B). With the exception of 0.08 mM, all Na-SA treatments increased the pH of HLB-infected citrus leaves. The concentrations higher than 0.14 mM all increased the pH (6.1 to 6.4). However, the numeric increase in pH (6.45) was highest with 0.14 mM Na-SA.

FIELD EXPERIMENT. Sodium salicylate applied to HLB-infected citrus trees increased the leaf sap pH from 6.0 to 6.1 (0.10 mM Na-SA) and 6.2 (0.14, 0.18, and 0.22 mM Na-SA) 1 d after its application (Fig. 7A). The pH increased to more than 6.3 after 4 d with the Na-SA concentrations of 0.14 and 0.22 mM. At 7 and 10 d also, with the exception of 0.10 mM, all Na-SA treatments increased leaf sap pH to 6.3 and 6.4. However at 14 d, the pH increased to 6.5 with 0.14 mM Na-SA. The differences between the ELP of different treatments were nonsignificant. Therefore, the data for ELP are not presented. The decrease in ELP of HLB-infected leaves was observed at 7 d post spray only, when the 0.18 and 0.22 mM treatments had the lowest ELP.

Field application of Na-SA at 0.10, 0.14 or 0.18 mM concentrations enhanced the growth of young foliage on HLB-infected citrus trees compared with the trees sprayed with DI water only (Fig. 7B). However, the highest Na-SA concentration (0.22 mM) did not induce young foliage growth. Sodium salicylate application also induced flowering in HLB-infected trees. The scores for flowering were highest for the 0.10 mM and lowest for 0.22 mM Na-SA treatments. The pictures of HLB infected citrus trees sprayed with water only (Fig. 8A) and 0.18 mM Na-SA (Fig. 8B) clearly show that SA enhanced the growth of new foliage and induced flowering.

Discussion

This study substantiates the protective effects of SA against heat, cold, and disease stresses in citrus. Our results show that endogenous application of Na-SA at appropriate concentrations stabilized the membrane properties of citrus plants exposed to different stress conditions. Electrolyte leakage, a rapidly measured parameter, provided evidence that SA protected membranes during both heat and cold stresses. Sodium salicylate applied to HLB-infected trees increased plant pH and stimulated new foliage growth and flowering.
Fig. 5 Effect of different concentrations of Na-SA on (A) electrolyte leakage and (B) freezing points of cold stressed citrus leaves at different times after Na-SA application. Values are means, vertical bars represent SE of means (n=4).

Table 1 Electrolyte leakage of cold stressed citrus leaves at different times after Na-SA application.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>1 d after Na-SA spray</th>
<th>7 d after Na-SA spray</th>
<th>14 d after Na-SA spray</th>
<th>21 d after Na-SA spray</th>
<th>28 d after Na-SA spray</th>
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<tr>
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<td>0.20</td>
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<td></td>
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<td>Concn (mM)</td>
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<td>-5.6</td>
<td>-4.4</td>
<td>-3.3</td>
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<tr>
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<td>56.2 c</td>
<td>49.7 a</td>
<td>43.8</td>
<td>45.9 a</td>
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</tr>
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<td>0.017</td>
<td>NS</td>
<td>NS</td>
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</table>

*aWithin column mean values followed by the same letter are not significantly different (P < 0.05) (n = 4).*
As indicated by ELP, heat stress damaged cell membranes of citrus leaves when no SA was applied. However, pretreatment with Na-SA at appropriate concentrations reduced the leakage of electrolytes from leaf cells. Due to an excessive excitation of the respiratory and photosynthetic electron transport systems, high temperature increases the production of reactive oxygen species (Dat et al., 1998b), which are toxic to plants. Lipid peroxidation of cellular and organelle membranes further leads to alterations in membrane fluidity and permeability. High temperatures can also cause direct heat injury to the plasma membrane and leakage of electrolytes from the cells (Ingram and Buchanan, 1981). However, exogenously applied SA can induce thermotolerance by enhancing antioxidant enzyme activity and preventing heat induced oxidative damage (Dat et al., 1998a). Foliar applications of 1 mM SA induced heat tolerance in cucumber plants as shown by the lower ELP, H$_2$O$_2$, and lipid peroxide levels (Shi et al., 2006). Exogenously applied SA has been used to induce heat tolerance in a variety of species like beans, tomato, potato, and Arabidopsis (Larkindale and Knight, 2002). However, SA applied at higher concentration (1 mM) had no protective effect in heat-stressed mustard seedlings due to decrease in catalase activity and increase in H$_2$O$_2$ content (Dat et al., 1998b, 2000). In our study, 0.10 to 0.20 mM Na-SA was the optimum concentration range to alleviate heat stress in citrus. The concentrations higher than 0.2 mM did not prevent citrus leaf cell damage. Maximum thermotolerance induction using SA at 0.1 mM has been previously reported in mustard seedlings (Dat et al., 1998b) and grapes (Wang and Li, 2006).

![Fig. 6.](image)

Fig. 6. (A) Electrolyte leakage and (B) leaf sap pH of healthy (Control) and HLB infected citrus leaves sprayed with different concentrations of Na-SA. (Values are means; vertical bars represent SE of means (n=6).)

![Fig. 7.](image)

Fig. 7 Effect of different concentrations of Na-SA on (A) leaf sap pH of huanglongbing (HLB) infected citrus trees at different times after Na-SA application and (B) young foliage growth and flowering score ratings for HLB infected citrus trees three weeks after Na-SA application. Young foliage growth score ratings are on the scale of 0 to 10 and flowering scores are on the scale of 0 to 5. Values are means, vertical bars represent SE of means (n=6).
Cold stress also resulted in considerable cell membrane damage to citrus leaves. However, Na-SA reduced ELP and maintained cell integrity in cold stressed citrus leaves. Cold stress disrupts and damages cell membranes, altering their permeability, and resulting in a loss of solutes or electrolytes. Freeze injury in citrus leaves is linearly related to the cellular dehydration resulting from extracellular freezing (Yelenosky and Guy, 1989). Chilling can lead to an increased concentration of toxic oxygen compounds in susceptible plant tissue (Hodgson and Raison, 1991). Therefore, reactive oxygen species are considered to be responsible for cold-induced injury as they may initiate degradative reactions, causing lipid peroxidation and membrane deterioration (Kang et al., 2003). Cold tolerance induced by SA is associated with an increase in the activity of antioxidant enzymes (Kang and Saltveit, 2002). Increase in cold tolerance with exogenous application of SA has been reported in maize (Janda et al., 1999), corn leaf, rice leaf, and cucumber hypocotyls (Kang and Saltveit, 2002), and grape plants (Wang and Li, 2006).

Sodium salicylate also lowered the freezing point of citrus by 1.4 to 2.5 °C. Non-acclimated citrus leaves survive low temperatures up to –4.4 °C, however the freezing points for citrus vary from –4.4 to –8.9 °C (Wiltbank and Oswalt, 1984). After 28 d following Na-SA spray, the freezing point of the control treatment was also lowered compared with 7, 14, and 21 d following spray. This could be due to cold acclimation because the temperatures during the experiment decreased from 22 to 8.2 °C (day 1 to 28). Citrus trees have the capability to develop considerable cold hardiness or cold acclimation if exposed to lower temperatures (Wiltbank and Oswalt, 1984; Yelenosky and Guy, 1989). This process of cold acclimation starts with cessation of growth and 12.8 °C is the threshold temperature generally used to induce cold hardiness in citrus (Rouse et al., 1977).

Huanglongbing-infected citrus trees had lower pH than healthy trees. Plant pH is an indicator of health and nutrient balance. The pH of the cell sap changes in response to imbalances in cation and anion uptake, because plant pH increases with the absorption of cations and decreases with absorption of anions (Hiatt, 1967). Therefore, at lower pH (<6.4) there is a deficiency of cations like calcium, magnesium, potassium and/or sodium, and at higher pH (>6.4) the anions like nitrogen, phosphate or sulfur are deficient. Several studies have documented the relationship between disease symptoms, plant tolerance, plant health, and plant pH (Noyes and Hancock, 1981). The variation in pH values with varying wheat and corn vigor has been reported previously in which resistant varieties had low acidity (Hurd-Karrer, 1925). Another study showed that mosaic-infected tobacco leaves had higher H+ concentration than healthy plants (Harvey, 1920). The pH of sunflower plants infected with Sclerotinia wilt was one unit lower than healthy plants due to high oxalic acid in the diseased plants (Noyes and Hancock, 1981). Pathogen infection alters the plant’s physiology, particularly the uptake, transport, and use of mineral nutrients (Schumann et al., 2010). Huanglongbing infection in citrus restricts the nutrient uptake and transport. Therefore, the differences in pH of healthy and diseased plants can be due to mineral imbalance. Huanglongbing-infected trees are lower in Ca, P, S, Zn, Mn, Fe, and Cu than healthy citrus trees (Spann et al., 2010 unpublished data). Calcium content of HLB-symptomatic trees has been reported to be as much as 50% lower than that of healthy citrus trees (Aubert, 2008). In addition, Ca plays an important role in plant defense pathways (Lecourieux et al., 2006) and Ca-deficient plants are more acidic than healthy plants receiving adequate Ca nutrition (Nightingale et al., 1931). A progressive decrease in the rate of tomato bacterial canker (Corynebacterium michiganense, a phloem limited bacterium) development was related to increased nutrient concentration and the rate of disease development was higher in K-deficient or

The present study showed that Na-SA application increased the pH of HLB-infected citrus trees and a concentration range of 0.14 to 0.18 mM Na-SA proved optimum to alleviate the detrimental effects of HLB. The effect of SA on plant pH can be due to improved nutrient uptake and plant health. Salicylic acid application has been reported to increase N, P, K, Ca, and Mg contents of maize plants along with increasing crop yield (El Khalil et al., 2009). Salicylic acid may also improve the health and vigor of plants by improving root growth. Foliar application of SA to the shoots of soybean plants significantly affected root size (Gutierrez-Coronado et al., 1998). Increased root growth has also been observed using SA concentrations of 10^{-10} M in *Tagetes erecta* (Sandoval-Yepiz, 2004). Earlier studies indicated that exogenous SA treatments stimulated root formation and increased mineral uptake by plants (Khan et al., 2003). Exogenously applied SA is also known to enhance CO_{2} assimilation and photosynthetic rate, thus improving plant health (Khan et al., 2003).

Sodium salicylate applied to HLB-infected citrus trees also induced young foliage growth and flowering. Salicylic acid and other salicylates play a regulating role in plant growth and productivity (Hayat et al., 2010). Exogenously applied SA has been reported to increase number of leaves, leaf area, and dry mass in corn and soybean (Khan et al., 2003). Salicylic acid applied at low concentrations (10^{-10} M) increased the shoot biomass and improved floral characteristics of *T. erecta* (Sandoval-Yepiz, 2004). Previous studies documented that SA regulated flowering in Lemma plants (Cleland and Ajami 1974). Salicylic acid sprayed at 100 μM concentration controlled flowering time in Arabidopsis (Martinez et al., 2004) and applied at 10 μM concentration stimulated flowering in *Lemma paucicostata* LP6 (Khurana and Cleland, 1992). Our results suggest that exogenous application of Na-SA can be a mechanism that integrates environmental signals with endogenous development of signals to regulate young foliage and flowering time. Therefore, a fine-tuned regulation of leaf flush and flower promotion pathways using SA can ensure the young foliage emergence and flowering at the proper time of the year in HLB-infected citrus trees.

In conclusion, our results provide evidence that exogenously applied SA can prevent the cell membrane damage caused by heat and cold stresses in citrus. In addition, Na-SA applied to HLB-infected citrus trees can raise plant pH, enhance new foliage growth, and stimulate flowering. Furthermore, the results revealed that the preventive effects of SA under different stress conditions are concentration dependent. A narrow concentration range of 0.10 to 0.20 mM Na-SA is most effective in protecting citrus from various stresses.

**Literature Cited**


Martinez, C., E. Pons, G. Prats, and J. Leon. 2004. Salicylic acid regulates...


