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EFFECT OF SALICYLIC ACID ON H_2O_2 METABOLISM DURING GRAPEFRUIT (*CITRUS PARADISI* MACFAD.) - *XANTHOMONAS CITRI* SUBSP. *CITRI* INTERACTION

SUMMARY

Citrus canker (*Xanthomonas citri* subsp. *citri: Xcc*) is a serious disease that causes significant yield and economic losses worldwide. The endogenous plant growth regulator salicylic acid (SA) was applied exogenously to canker-infected grapefruit (*Citrus paradisi* Macfad.) at 500 µM to determine its effects on oxidative metabolism. Initial visual symptoms during canker development were water soaking at 2 days after inoculation (dai) followed by hypertrophy and ruptured epidermis between 8-10 dai with the formation of tiny yellow-colored bacterial colonies. *Xcc* depressed hydrogen peroxide (H_2O_2) levels in leaves accompanied by reduced superoxide dismutase activity (SOD: EC 1.15.1.1) and higher catalase (CAT: EC 1.11.1.6), ascorbate peroxidase (APOD: EC 1.11.1.11), and guaiacol peroxidase (POD: EC 1.11.1.1) activities. The exogenous application of SA did not affect *Xcc* population or canker development, however, levels of SOD and POD were elevated and persisted during pathogenesis. SA alone and in association with *Xcc* suppressed H_2O_2. SA restored the levels of SOD and POD, but CAT and APOD activities contributed to a substantial decline in H_2O_2 levels and maintained a favorable environment for *Xcc* growth.

Keywords: oxidative stress, superoxide dismutase, catalase, ascorbate peroxidase, peroxidase.

INTRODUCTION

Citrus grapefruit (*Citrus paradisi* Macfad.) is a commercially important crop grown in more than 74 countries worldwide (Talon *et al* 2008). Despite being economically significant grapefruit are highly susceptible to canker [*Xanthomonas citri* subsp. *citri* (*Xcc*); Gottwald *et al* 2009] often causing production losses (Anonymous 2009). Cankers infects all aboveground plant parts and appear as raised, brown colored lesions surrounded by yellow halos on foliage (Gottwald *et al* 2009). Heavy infestation can cause leaf abscission and premature fruit drop (Gottwald *et al* 2009).

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Several approaches have been adopted in Florida to control canker outbreaks. Initially, infected and surrounded uninfected trees were removed to eradicate the pathogen (Schubert *et al* 2001). Once eradication was deemed ineffective, various combinations of copper (Cu) based formulations became the standard control method (Behlau *et al* 2008). Although, Cu has been an effective measure against canker, long-term use may cause phytotoxicity and be an environmental hazard (Alva *et al* 1995). In addition, continuous Cu exposure has promoted Cu resistant *Xcc* populations (Rinaldi *et al* 2000).

Salicylic acid has been shown to induce local and systemic acquired resistance (SAR) upon pathogen recognition through elevated concentration at the site of infection in several plant-pathogen interactions (Malamy *et al* 1990). The exogenous application of SA is known to induce synthesis of pathogenesis-related protein and maintain elevated levels of H_2O_2 during pathogen challenge in various plant species (Park *et al* 2009). Interactions between SA and H_2O_2 are quite complex and both can induce the synthesis of each other (Vlot *et al* 2009). Physiological significance of these potentially interacting signaling molecules remains largely unknown during canker development in citrus.

H_2O_2 is a reactive molecule and can induce defense related genes during pathogen attack in plants (Lamb and Dixon 1997). H_2O_2 is also antimicrobial and has been shown to impede growth of *Xcc* populations *in vitro* (Tondo *et al* 2010) and *in vivo* (Kumar *et al* 2011a) in canker resistant kumquat (*Fortunella margarita* (Lour.) Swingle). The steady state levels of H_2O_2 in plants are predominantly governed by the rates of production and degradation of various enzymatic and non-enzymatic pathways (Mittler 2002). Superoxide dismutases are mainly responsible for the generation of H_2O_2 in *Xcc* inoculated leaves of kumquat, which is an early event of the hypersensitive response (HR) and critical for the isolation of *Xcc* by inducing plant cell death (PCD; Kumar *et al* 2011a). Since the levels of H_2O_2 and SOD activity declined in *Xcc* challenged grapefruit leaves (Kumar *et al* 2011b), it can be hypothesized that exogenous application of SA can be beneficial in restoring the compromised defense system.

The mode of action of SA in disease resistance is still largely unknown. However, studies have shown that SA inhibits the activity of H_2O_2 degrading enzymes CAT and APOD, which promote higher concentrations of H_2O_2 during pathogen attack (Chen *et al* 1993; Dempsey *et al* 1999), but this mode of action is not universal as physiological concentrations of SA < 1 mM were not sufficient to inhibit the CAT activity in several plant *sp.* (Bi *et al* 1995; Guan and Scandalios 1995). SA can generate $O_2^{\cdot-}$ radicals in conjunction with apoplastic guaiacol peroxidases and H_2O_2 (Kawano *et al* 1998). Guaiacol peroxidases are class III PODs and perform a wide variety of physiological functions (Kawano *et al* 1998). PODs are generally considered antioxidant enzymes but under certain conditions generate significant quantities of reactive oxygen species (ROS; Kawano *et al* 1998). In our initial study with an exogenous spray application of SA (5 mM), we found no effect on the *Xcc* population and symptom development (Kumar *et al* 2011c). The present investigation was conducted with simultaneous

infiltration of SA 500 µmole and *Xcc* (≈ 10^6 cfu cm^{-2}) in grapefruit leaves to determine whether SA can restore host cell oxidative metabolism including enzyme activities of SOD, CAT, APOD, and POD and suppress *Xcc* populations and disease symptoms.

MATERIAL AND METHODS

Plant material and culture

'Grapefruit' plants were purchased from a commercial nursery (Harris Citrus Nursery, Lithia, FL, USA) and maintained in 15 cm x 8 cm plastic pots containing potting mix Fafard Mix 4P (Southern Agricultural Insecticides Inc., Palmetto, FL, USA). Mineral nutrition was provided periodically using Peters' professional fertilizer (The Scott Co., Marysville, OH, USA). Two year old green house grown plants were acclimatized for 60 days in environmental growth chambers at temperatures ranging from 25°-28°C, and relative humidities maintained at 65-95% before sampling. The inoculum used in this study was the *Xanthomonas citri* subsp. *citri*-NK-08 strain (*Xcc*) isolated from a foliar lesion on a Valencia orange tree located in a commercial citrus grove near Immokalee, FL, USA and was confirmed using an *Xcc* Immuno Strip (Agdia Inc., Elkhart, IN, USA). The strain was stored in 10% glycerol (v/v) at –80°C. Bacterial cultures were streaked to nutrient agar (Difco Laboratories, Detroit, MI, USA) prior to use. To produce inocula, bacteria were transferred from nutrient agar to a 1000 ml flask containing 700 ml nutrient broth (Difco Laboratories) and grown at 28°C for 24 hr in a shaker incubator (C24 incubator Shaker, New Brunswick Scientific, Edison, NJ, USA) at 125 rpm. Cells were harvested in the late log phase and adjusted to a final concentration of 10^8 cfu ml^{-1} using a spectrophotometer (Bio-Spec Mini; Shimadzu, Tokyo, Japan) at 600 nm. The bacterial concentration was further confirmed by counting colonies in serial dilutions of the bacterial suspension on nutrient agar after 3 d. *Xcc* populations at different stages of growth viz., lag phase, early exponential phase, late exponential phase, and stationary phase were incubated for 30 min in different concentrations of H_2O_2 (0.25, 1, 3, and 5 mM) and SA (0.5, 1, 3, and 5 mM) for 2 hr and on SA containing plates (0.5, 1, 3, and 5 mM) for 72 hr. After incubation period of 30 min in H_2O_2 and 2 hr in SA aliquots were plated on nutrient agar plates and incubated at 28°C (Tondo *et al* 2010). The colonies were counted after 3 d and used to calculate mean cfu ml^{-1}. Basal mature leaves (fully expanded) were inoculated using a 1 ml tuberculin syringe without a needle (Viloria *et al.*, 2004). *Xcc*, 500 µM SA, *Xcc*+500 µM SA, and sterile nutrient broth (control) were infiltrated slowly into the abaxial surface of leaves on both sides of the midrib to produce a 22-25 mm diameter water soaked zone that contained approximately 40 µl of injected solution. We infiltrated 500 µM SA since it maintained relatively higher concentrations of endogenous H_2O_2 compared to 250 and 1000 µM SA concentrations (unpublished data) when infiltrated simultaneously with *Xcc* in grapefruit leaves. Five plants each were used for each treatment with several leaves injected with the same treatments. Three basal

leaves were removed per plant at each sampling time from five plants and two 7 mm diameter leaf disc from each leaf were ground in 1 ml of sterile water. Ten-fold serial dilutions from of each sample were plated on nutrient agar plates and incubated at 28°C. The colonies were counted after 3 d and used to calculate mean cfu cm^{-2} for each leaf (Lund *et al.*, 1998). Leaf samples (0.5 g) were homogenised in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 x g for 15 min at 4°C. The supernatant (0.3 ml) was mixed with 1.7 ml potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide (KI) solution and incubated for 5 min before measuring the absorbance at 390 nm using a Bio-Spec Mini spectrophotometer (Shimadzu, Tokyo, Japan; Velikova *et al* 2000). Leaf samples (0.5 g) were homogenised in 100 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 2% (w/v) polyvinylpyrrolidone (PVP) in a pre-chilled pestle and mortar. The extraction buffer also contained 5 mM ascorbate and the homogenate was centrifuged at 15,000 x *g* at 4°C for 30 min. For SOD (EC 1.15.1.1) analysis the supernatant was filtered through a Sephadex G-25 PD-10 gel column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The assay solution (total volume 3.0 ml) contained 201 mM methionine, 1.72 mM nitrobluetetrazolium (NBT), an appropriate aliquot (10-25 µl) of crude leaf extract, 50 mM potassium phosphate buffer (pH 7.8), and 0.12 mM riboflavin, with the riboflavin added last. The assay tubes were shaken and placed 30 cm below a light source consisting of two 75 W fluorescent bulbs. The reaction was started by switching on the light and allowed to run for 10 min. The tubes were covered with black cloth immediately after switching-off the light. Non-irradiated reaction mixtures containing the same leaf extract, which did not develop any colour, were used as controls. Blanks without leaf extract developed the maximum colour. Absorbance was measured at 560 nm. One unit of SOD activity was defined as the enzyme activity which caused a 50% inhibition in the initial rate of reaction measured in the absence of extract (Dhindsa *et al* 1981). CAT (EC 1.11.1.6) activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and enzyme. The decomposition of H_2O_2 was followed at 240 nm (extinction coefficient = 39.4 mM cm^{-1}; Cakmak and Marschner, 1992). APOD (EC 1.11.1.11) activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1.0 mM H_2O_2, 0.25 mM ascorbic acid and enzyme. The activity was determined by the rate of ascorbate oxidation at 290 nm (extinction coefficient = 2.8 mM cm^{-1}; Nakano and Asada 1981). Protein content was determined using BSA as the standard (Bradford 1976). POD (EC 1.11.1.1) activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, 0.05 % guaiacol and enzyme. The activity was determined by the increase in absorption at 470 nm due to guaiacol oxidation (extinction coefficient = 26.6 mM cm^{-1}; Cakmak and Marschner 1992). The experiment was conducted as a randomized complete-block design with five blocks, and one plant of each *Xcc* treatment per block in a growth chamber. Five

plants were used for each treatment. On each sampling date, three leaves per plant were removed and all assays were conducted on each leaf, which represented five replicates (plants) and each replicate with three sub-samples (leaves). Two independent experiments were conducted. Mean values per plant were determined and subjected to analysis of variance (SAS Institute, Cary, NC, USA) with means separated using a protected least significant difference (LSD) at $P < 0.05$. The standard error (SE) of the mean was also calculated. Two independent experiments were conducted for *in vitro* growth of *Xcc* and completely randomized design was used for analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Following *Xcc* infiltration water soaking was the first visual symptom observed at 2 dai and became more pronounced by 4 dai (Figure 1). The abaxial and adaxial epidermis of the *Xcc*-infiltrated region was raised, ruptured, and thickened into a spongy mass by 8 dai. Yellow colored bacterial colonies were visible 10 dai on the upper epidermis. No differences in symptom development were observed between the *Xcc* and *Xcc*+500 µM SA treatments.

Fig. 1 Symptom develoment in *Xcc*-infected leaves of grapefruit. The number at the top of each leaf indicates days after inoculation (dai).

The *in vivo Xcc* population increased from 3.7 x 10^6 cfu cm^{-2} at 0 dai to 9.5 x 10^8 and 9.9 x 10^8 cfu cm^{-2} in *Xcc* and *Xcc*+500 µM SA infiltrated leaves, respectively by 10 dai (Figure 2A). Bacterial population was in the log phase of growth 0-2 dai and thereafter the stationary phase. The *Xcc* population increased ≈10-fold from 0 to 1 dai, and ≈1000-fold from 0 to10 dai in both *Xcc* and *Xcc*+500 µM SA treated leaves.

SA 0.5 mM did not affect *Xcc* growth from the lag phase to late log phase of growth (Table 1). However, SA at 3 and 5 mM concentration significantly reduced *Xcc* growth during the stationary phase.

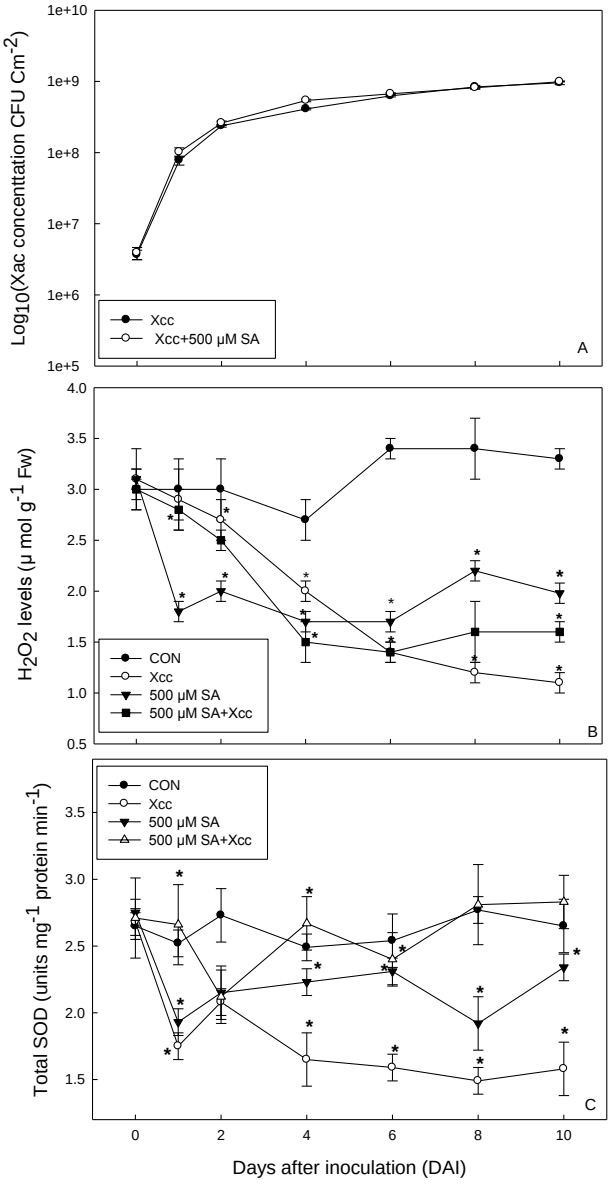

Fig. 2 Mean values (n=15) of *Xcc* populations (Panel A), H_2O_2 concentration (Panel B), and superoxide dismutase activity (Panel C) at various times (days after inoculation; dai) with *Xanthomonas citri* subsp. *citri* (*Xcc*). Vertical bars represent SE unless smaller than the data symbol. Asterisks indicate these mean values that are significantly different from the controls (means separated using LSD at $P < 0.05$).

Table 1 *In vitro Xcc* populations$_{log10}$ (cfu ml^{-1}) after 2 and 72 hr exposure to different concentrations of salicylic acid

Phases (2 hr)	Control	0.5 mM SA	1 mM SA	3 mM SA	5 mM SA
Lag	9.5 (±1.0)	9.5 (±1.1)	9.7 (±1.5)	9.6 (±1.4)	9.6 (±1.6)
Early Log.	11.5 (±2.2)	11.8 (±2.3)	11.6 (±1.2)	11.8 (±1.5)	11.5 (±1.7)
Late Log.	12.5 (±1.2)	12.6 (±2.2)	12.6 (±1.5)	12.8 (±1.6)	12.3 (±1.5)
Stationary	11.6 (±1.5)	11.2 (±1.3)	11.1 (±1.0)	10.9 (±1.2)*	10.5 (±1.0)*
Phases (72 hr)					
Lag	9.3 (±1.2)	9.4 (±1.4)	9.2 (±1.1)	8.8 (±1.5)*	7.3 (±1.0)*
Early Log.	11.9 (±1.1)	11.8 (±1.3)	10.1 (±0.8)*	9.7 (±1.0)*	7.4 (±1.1)*
Late Log.	13.5 (±1.9)	13.2 (±1.3)	12.9 (±1.0)	10.7 (±1.3)*	10.9 (±1.4)*
Stationary	12.8 (±1.3)	12.7 (±1.4)	12.3 (±1.3)	10.6 (±1.1)*	10.4 (±1.4)*

The standard error (± SE) of each mean (n = 10) was calculated and asterisks indicate the mean values that are significantly different from the controls (LSD, $P < 0.05$).

Table 2 *In vitro Xcc* populations$_{log10}$ (cfu ml^{-1}) after 30 min exposure to H_2O_2

Phases	Control	0.25 mM H_2O_2	1mM H_2O_2	3mM H_2O_2	5 mMH_2O_2
Lag	10.79 (±1.2)	10.49 (±1.3)	9.01 (±0.9)*	6.83 (±1.4)*	5.07 (±1.4)*
Early Log.	11.88 (±2.2)	10.26 (±1.5)*	9.18 (±1.3)*	7.86 (±2.3)*	7.12 (±2.2)*
Late Log.	13.52 (±2.4)	12.38 (±1.2)*	11.08 (±1.3)*	11.19 (±1.8)*	10.63 (±1.9)*
Stationary	12.71(±2.1)	12.54 (±3.2)	11.44 (±2.3)*	11.45 (±2.2)*	11.58 (±2.5)*

The standard error (± SE) of each mean (n = 10) was calculated and asterisks indicate the mean values that are significantly different from the controls (LSD, $P < 0.05$).

The long term exposure of SA for 72 hr caused 99% inhibition in population growth between the lag and stationary phase (Table 1). *Xcc* populations showed 0.8% survival at the lag phase, 0.02% at the early exponential phase, 0.7% at late exponential phase and maximum (0.9%) survival was observed during stationary phase at 5 mM SA concentration. All the growth phases were susceptible for long term exposure to SA than short term 2 hr exposure.

In vitro H_2O_2 had a direct antimicrobial effect on the *Xcc* population (Table 2). Among the different growth phases the lag, early exponential and late exponential phases were more susceptible to H_2O_2. However, the stationary phase *Xcc* populations were relatively more resistant to H_2O_2. Increasing concentrations of H_2O_2 exerted more inhibitory effects on the growing *Xcc* populations. Only 0.0002% *Xcc* survival was observed at the lag phase, 0.002% at the early exponential phase, 0.13% at the late exponential phase, and 25.3% at the stationary phase when exposed to 5 mM H_2O_2.

Following *Xcc* inoculation, levels of H_2O_2 declined progressively in *Xcc*, and *Xcc*+500 µM SA treatments (Figure 2B). A similar trend was observed when leaves were infiltrated with 500 µM SA alone. The levels of H_2O_2 declined from 3.1 µmol g^{-1} fw to 1.1: *Xcc*, 1.6: *Xcc*+500 µM SA, and 1.98 µmol g^{-1} fw in 500 µM SA infiltrated leaves. The decline was 64.5%: *Xcc*, 46.6%: *Xcc*+500 µM SA, and 36.1% in 500 µM SA treated leaves from 0 to 10 dai.

Superoxide dismutase activity remained unchanged in control leaves but was progressively reduced in *Xcc*-infected plants (Figure 2C). SOD levels were also lower in the 500 µM SA infiltration treatment. However, simultaneous infiltration of *Xcc*+500 µM SA restored the levels of SOD activity compromised in *Xcc* and 500 µM SA treatments. The rise in SOD activity was 1.5-fold: 1 dai, 1.6-fold: 4 dai, 1.9-fold: 8dai, and 1.8-fold at 10 dai compared to *Xcc*-infected leaves.

Catalase activity was constant up to 10 dai in control leaves (Figure 3A). CAT in *Xcc*-infected leaves was lower than the control 1 dai and thereafter was substantially higher than the controls. The rise in CAT activity was 1.4-fold at 2 dai, 2.6-fold (4dai), and 2.3-fold at 10 dai compared to activity at 0 dai. The highest activity was observed at 4 dai (0.044 µmol H_2O_2 min^{-1} mg^{-1} protein). Leaves infiltrated with 500 µM SA also showed higher CAT activity compared to control leaves. A slight decline in CAT activity was observed between 4-6 dai for the *Xcc*+500 µM SA treatment. However, these levels (0.027: 4 dai, 0.029 µmol H_2O_2 min^{-1} mg^{-1} protein: 6 dai) of CAT were higher than the controls (0.015: 4dai, 0.017 µmol H_2O_2 min^{-1} mg^{-1} protein: 6 dai).

Ascorbate peroxidase activity increased (49%) sharply at 1 dai and peaked at 2 dai (0.52 µmol asc. mg^{-1} protein min^{-1}) in *Xcc*-infected leaves (Figure 3B). The rise in APOD activity was 1.4-fold: 1 dai, and 1.6-fold at 4, 8, and 10 dai in comparison to control leaves. Moderate suppression (24%) in APOD activity was observed at 1 dai in 500 µM SA treatment in comparison to activity at 0 dai. Later APOD activity was higher than the controls. Only 14 and 16% suppression in APOD activity was observed at 1 and 2 dai respectively in *Xcc*+500 µM SA treatment in contrast to APOD levels in *Xcc* infiltrated leaves. APOD activity was 23.5% (1 dai) and 60% (2 dai) higher than controls. The peak of APOD activity was observed at 8 dai (0.50 µmol asc. min^{-1} mg^{-1} protein) in this interaction.

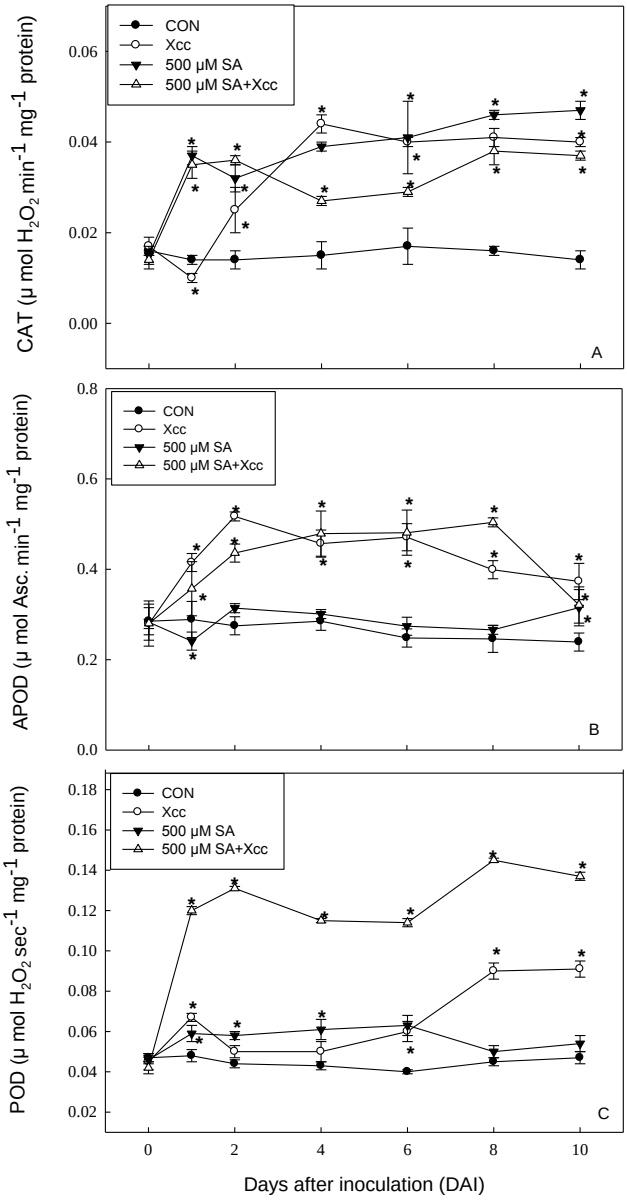

Fig. 3 Mean values (n=15) of catalase activity (Panel A), ascorbate peroxidase (Panel B), and peroxidase (Panel C) at various times (days after inoculation; dai) with *Xanthomonas citri* subsp. *citri* (*Xcc*). Vertical bars represent SE unless smaller than the data symbol. Asterisks indicate these mean values that are significantly different from the controls (means separated using LSD at $P < 0.05$).

Guaiacol peroxidase activity increased in all the treatments except controls, where POD activity maintained at constant levels (Figure 3C). Levels of POD were higher in *Xcc* and 500 µM SA treated leaves in comparison to control leaves. The largest surge in POD activity was noted in the *Xcc*+500 µM SA treatment between 1 to 10 dai. The peak of this activity (0.145 µmol H_2O_2 sec^{-1} mg^{-1} protein) was observed at 8 dai and was highest among all the treatments with a rise of 222% over the controls.

DISCUSION

Canker development in citrus grapefruit is governed by a sequence of highly organized events (Kumar *et al* 2011b). The first visual sign of canker infection is the appearance of water soaking at 2 dai (Figure 1). The molecular and cellular basis for water soaking could be explained on the basis of attachment of the type III secretion system by hrp (hypersensitive response and pathogenecity) pili to mesophyll cells and secretion of effector proteins pthA (pathogenicity), Avr (avirulence), and Pop (pseudomonas outer protein) that in turn promote hypertrophy (cell elongation) and hyperplasia (cell division) in host tissue (Figure 1; Brunings and Gabriel 2003). The rapid rate of cell division and enlargement of plant cells results in loss of intercellular space and generates capillary mediated water uptake, which appears as water soaking (Popham *et al* 1993). *Xcc* induced accelerated cell division and elongation of mesophyll cells (Brunings and Gabriel 2003) in association with higher water uptake provided sufficient intercellular pressure to rupture the epidermis by 8 to 10 dai, which coincided with the stationary phase population of *Xcc* ($\approx 10^9$ cfu cm^{-2}) that later progressed towards the death phase (Figure 2A; Kumar *et al* 2011b).

In Vivo, ≈ 1000-fold increase in *Xcc* populations were observed in *Xcc* and *Xcc*+500 µM SA treatments from 0 to 10 dai. No adverse or antimicrobial effects of SA treatments were observed on growing *Xcc* populations at various stages of growth and canker development. Population growth was similar in both treatments. These observations are contradictory to several reports that strongly support the anti-bacterial nature of endogenous SA and its direct effect on pathogens, although the reported bacteria are in a different genus including *Pseudomonas aeruginosa* (Prithiviraj *et al* 2005a), and *Staphylococcus aureus* (Prithiviraj *et al* 2005b). Intercellular washing fluid collected from age related resistance expressing Arabidopsis plants contained higher levels of SA (0.001 to 0.02 mM) and negatively affected the growth of *Pseudomonas syringae* (Cameron and Zaton 2004). Successful growth of *Xcc* under 500 µM (0.5 mM) SA treatment might be attributed to endogenous concentrations of SA that might be too low to exert any direct killing effect and exogenous treatment of SA could induce higher activity of SA-inducible glucosyltransferase (SA-GTase) and significantly reduce the levels of free SA in the zone of host-*Xcc* interaction. A seven fold rise in SA-GTase activity was observed 6 hour post infiltration of 1 mM SA in rice seedlings (Silverman *et al* 1995). Considering the occurrence of these possibilities in grapefruit-*Xcc*-SA interactions, we further tested the

antibacterial role of SA *in vitro* to determine whether the anti-bacterial effect of SA is concentration or time dependent (Table 1). Interestingly, *in vitro* data confirmed that SA had no killing effect on *Xcc* populations under physiological concentrations of SA during short and long term exposure. However, 99% inhibitions in stationary phase *Xcc* populations were observed under 5 mM SA regime during long term exposure. Moreover, 5 mM SA spray application failed to control the *Xcc* populations in grapefruit leaves (Kumar *et al* 2010c). Direct killing effects of SA on pathogens are rare under physiological concentrations. In the case of *Xcc*, SA is antimicrobial only under non-physiological concentrations and requires long term exposure, which is not feasible as a control measure for citrus canker in commercial groves.

The main objective of this work was to explore the role of SA in citrus and *Xcc* with regards to oxidative metabolism in plant defense. *In vitro* experiments were conducted to gain insight into the detrimental effects of H_2O_2 on *Xcc* populations. *Xcc* is a strict aerobe and constantly exposed to peroxidative challenge either by its own metabolic activities or through the host's reaction during pathogenesis (Tondo *et al* 2010). Different growth phases of *Xcc* have variable susceptibility levels for H_2O_2, early growth phases: lag and log are more susceptible than the stationary phase (Table 2). Higher levels of H_2O_2 had more inhibitory effects on population growth. Only 0.0002% *Xcc* survival was observed at the lag phase of growth and that enhanced to 25.3% during stationary phase under 5 mM H_2O_2 exposure. The phase specific H_2O_2 resistance is governed by the differential expression of *Xcc* antioxidant enzyme catalases (Tondo *et al* 2010). The endogenous levels of H_2O_2 were modified by *Xcc* in grapefruit leaves (Figure 2B). Levels declined by 64.5% by the end of the study (10 dai), which reflects the evolution of a crucial strategy in *Xcc* to avoid peroxidative challenge. A similar decline (46.6%) was also found in *Xcc*+500 µM SA treated plants but the decline was less compared to *Xcc*-treated plants. SA promoted higher levels of H_2O_2 but they still were lower (2.0-fold) than controls to impart any adverse effect on the *Xcc* population. However, these modified levels might be useful in signal transduction that induces defense related gene expression (Lamb and Dixon 1997). SA alone reduced the levels of H_2O_2 in citrus grapefruit by maintain low SOD and CAT activity (Figure 2C, 3A).

It has been shown that SODs are essential for generation of H_2O_2 during citrus-*Xcc* interaction (Kumar *et al* 2011a). Yet SOD levels declined by 69.9% between 0 to 10 dai in *Xcc*- infected grapefruit leaves and partly accounted for the lowering of H_2O_2 (Figure 2C). *Xcc* is well equipped to invade and suppress the first line of plant defense and maintain a favorable environment for survival and growth in grapefruit.

SOD activity increased under *Xcc*+500 µM SA treated plants. The rise was 1.5-fold higher at 1 dai and later exceeded the levels of controls at 4 (61.8%), 8 (88.5%), and 10 (79.1%) dai. The rise in SA mediated SOD activity is crucial at 1 dai compared to later stages as the lag and log phase of *Xcc*

populations are more susceptible to H_2O_2. The increase in Mn-SOD and Cu-Zn-SOD transcript was also observed upon treatment of SA and *Pseudomonas syringae* in *Nicotiana plumbaginifolia* (Bowler *et al* 1989). SOD operates in association with NADPH oxidase to produce H_2O_2 during HR (Lamb and Dixon 1997). Higher activity of SOD was accompanied by elevated levels of CAT (2.6-fold), APOD (1.1-fold), and POD (3.26-fold) between 0 to 10 dai, which finally quenched the levels of H_2O_2 by 46.6% (Figure 3A, B, C). SA induced higher SOD activity provide a new metabolic challenge to manipulate the activities of CAT, and APOD in citrus to confer a check on *Xcc* populations by enhancing the levels of H_2O_2.

Conflicting data is available in the literature regarding the effects of SA on CAT and APOD; negative as well positive effects were reported in different plant pathogen interactions (Chen *et al* 1993; Dempsey *et al* 1999). The activity of CAT showed a steep rise at 2 dai in *Xcc*-infected leaves of grapefruit (Figure 3A). In contrast CAT activity declined at 6 dai in *Xcc*-infected kumquat plants (Kumar *et al* 2011c).

SA application slightly declined the CAT activity at 4 (38.6%) and 6 (27.5%) dai in *Xcc*+500 µM SA treated plants but these levels were higher than controls by 48% (4 dai) and 70.5% (6 dai). Therefore, SA suppression of CAT was insubstantial and occurred only for a short duration while rest of pathogenesis was dominated by higher CAT activity in all treatments except controls and with a transient inhibition (28.5%) of CAT at 1 dai in 500 µM SA treated plants. Moreover, physiological concentrations of SA (< 1 mM) did not affect CAT activity in tobacco (Bi *et al* 1995). *Xcc* not only induced higher CAT activity in grapefruit but also induced pathogenesis related novel CAT isoforms (Kumar *et al* 2011b).

APOD is an important quencher of H_2O_2 (Mittler 2002). Apparently, APOD inhibition is crucial to enhance H_2O_2 levels during plant pathogenic interactions. Unfortunately, APOD activity increased in all treatments compared to controls with a slight declined at 1 (14%) and 2 (16%) dai for *Xcc*+500 µM SA treated plants compared to *Xcc*-infected plants. In addition, a moderate declined (24%) was observed 1 dai in the 500 µM SA treatment. These transient declines and thereafter a steep rise in APOD activity showed the reversible inhibition of APOD by SA (Durner and Klessig 1995). It has been reported that SA is not an effective inhibitor of cytoplasmic APOD (Tenhaken and Rubel 1997). However, strong suppression of APOD was not observed in the present investigation, which is required for a potent HR (Mittler *et al* 1998). We previously observed a significant decline in APOD activity 1 dai and at later stages of cell death in *Xcc*-infiltrated region of kumquat leaves (Kumar *et al* 2011d). APOD may be good a target in grapefruit for genetic manipulation through genetic engineering or conventional breeding programs using kumquat.

In addition to CAT and APOD, *Xcc* also controlled apoplastic guaiacol peroxidases, which belongs to class III of the peroxidase family (Kawano *et al* 1998). POD activity increased in all treatments except controls. Highest POD

activity was recorded for *Xcc*+500 µM SA treated plants. POD may generate hydroxyl radicals to cleave load bearing bonds of cell wall polymers and hence facilitate loosening of mesophyll cells for enlargement and division (Liszkay *et al* 2003). A quite opposite and restricted role of POD may be seen at the boundary of the infected region where higher POD activity enhanced the cross linking of the cell wall by lignin biosynthesis, and prevent pathogen spread beyond the area of inoculum infiltration, which was evident from the localization of canker (Bradley *et al* 1992). PODs are generally known for antioxidant properties but can generate substantial amounts of ROS (Kawano *et al* 1998).

In **conclusion**, on the basis of our findings we propose that SA partially interferes with the *Xcc* manipulated host machinery by increasing the activity of SOD and POD to raise H_2O_2 (Kumar *et al* 2011d). But *Xcc* as well as SA induced higher levels of CAT and APOD counteract the functions of SOD and POD by depleting the levels of H_2O_2.

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UTICAJ SALICILNE KISELINE NA METABOLIZAM H_2O_2 TOKOM INTERAKCIJE GREJPFRUT (*CITRUS PARADISI* MACFAD.) - *XANTHOMONAS CITRI* SUBSP. *CITRI*

SAŽETAK

Pjegavost citrusa (*Xanthomonas citri* subsp. *citri: Xcc*) je ozbiljno oboljenje koje uzrokuje značajan pad prinosa i ekonomske gubitke širom svijeta. Endogeni regulator rasta biljke salicilna kiselina (SA) primijenjena je egzogeno na grejprfut (Citrus paradisi Macfad.) zaražen pjegavošću u količini od 500 µM kako bi se utvrdio njen uticaj na oksidativni metabolizam. Početni vizuelni simptomi tokom razvoja pjega bili su izbijanje vode 2 dana nakon inokulacije, potom hipertrofija i rupture epidermisa u period između 8-10 dana sa formiranjem malih, žutih kolonija bakterija. *Xcc* je smanjila nivoe vodonik peroksida (H_2O_2) u lišću, što je praćeno smanjenom aktivnošću superoksid dismutaze (SOD: EC 1.15.1.1) i povećanom aktivnošću katalaze (CAT: EC 1.11.1.6), askorbat peroksidaze (APOD: EC 1.11.1.11) i gvajakol peroksidaze (POD: EC 1.11.1.1). Egzogena primjena SA nije imala uticaj na Xcc populaciju niti razvoj pjega; međutim, nivoi SOD i POD su bili povišeni i trajal su tokom patogeneze. Sama SA I zajedno sa Xcc potisnuli su H2O2. SA je povratila nivoe SOD i POD, ali su aktivnosti CAT i APOD doprinijele značajnom smanjenju nivoa H202 i održale povoljnu sredinu za rast *Xcc*.

Ključne riječi: oksidativni stres, superoksid dismutaza, katalaza, askorbat peroksidaza, peroksidaza.