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Responses of *Citrus medica* var. *sarcodactylis* during *Xanthomonas citri* subsp. *citri* Infection

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Asiatic citrus canker [*Xanthomonas citri* subsp. *citri*; (*Xcc*)] is a serious bacterial disease that can causes economic losses in citrus production and marketing throughout the world. In the present investigation, canker lesion development was examined in Buddha Hand (*Citrus medica* var. *sarcodactylis*), which is an ornamental tree. Basal matured leaves (fully expanded) were inoculated using a tuberculin syringe (1 cc) without a needle. Citrus canker inoculum (1.1×10^6 cfu/cm^2) was slowly infiltrated into the abaxial leaf surface on both sides of the mid vein to produce a zone of water-soaked tissue. The highest *Xcc* population (1.9×10^{11} cfu/cm^2) was observed at 8 days after inoculation (dai) and thereafter declined. This decline was associated with higher oxidative redox of host tissue. These results showed that Buddha Hand represents a delayed type of hypersensitive-like response. Buddha Hand may serve as an important citrus species for resistance to citrus canker that might be exploited through transgenic and hybrid plants.

Asiatic citrus canker [*Xanthomonas citri* subsp. *citri* (*Xcc*)] is an economically important disease that is caused by a gram negative obligate aerobic bacterium (Tondo et al., 2011a). The pathogen affects all plant parts and causes raised, brown, and corky lesions surrounded by water-soaking and yellow halos (Graham et al., 1992). Severe infection of the disease causes premature abscission of fruits and young leaves and thus reduces quality and quantity of fruit production (Francis et al., 2009; Schubert et al., 2001).

Commercially available citrus species are susceptible to citrus canker and uses of various control measures have only limited effect on disease (Deng et al., 2010). Search of canker-resistant genotypes with active resistance mechanisms would be beneficial for incorporating resistance mechanisms in susceptible genotypes either through breeding or genetic manipulation.

Deng et al. (2010) showed the existence of active canker resistance in Chinese citron (*Citrus medica*). These plants showed formation of necrotic lesions following inoculation of 2.5×10^7 cfu/mL of *Xcc* suspension. The mechanism of resistance is still unknown. In the present investigation, we explored the oxidative responses of an ornamental citron Buddha Hand (*Citrus medica* var. *sarcodactylis*) following *Xcc* inoculations. Our earlier studies on canker resistant kumquat revealed the accumulation of hydrogen peroxide (H_2O_2) upon *Xcc* infection and followed by a four-fold decline in *Xcc* populations (Kumar et al., 2011a).

The generation of reactive oxygen species (ROS) can be the first crucial event during plant–pathogen interactions (O'Brien et al., 2012). ROS includes superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$; Grant and Loak, 2000; Voludakis et al., 2006) and act as antimicrobial factors or intracellular or intercellular signaling molecules (O'Brien et al., 2012). Superoxide dismutases (SODs) are the primary components of oxidative metabolism, which convert superoxide radicals into H_2O_2 (Bowler et al., 1992). Hydrogen peroxide acts as a secondary messenger under low concentrations and antimicrobial under higher concentrations that promotes cell death of both host and *Xcc* (Kumar et al., 2011a; Lamb and Dixon, 1997).

Plants have developed strategies to accumulate higher levels of H_2O_2 by suppressing the activities of H_2O_2 catabolizing enzymes such as catalase and ascorbate peroxidase (Kumar et al., 2011b). Catalase activity was suppressed in *Xcc*-infected kumquat leaves 6 dai (Kumar et al., 2011b). Besides the importance of SOD and CAT in regulation of oxidative burst, the role of the class III cell wall peroxidase, guaiacol peroxidase (POD), is of utmost importance (O'Brien et al., 2012). Peroxidase-dependent oxidative bursts have been characterized in *Daucus carota* (Bach et al., 1993), *Arabidopsis thaliana* (Davies et al., 2006), and *Capsicum annuum* (Choi et al., 2007). PODs also contribute to multiple functions during pathogen attack such as lignification, suberization (Quiroga et al., 2000), and cross-linking of cell wall proteins (Showalter, 1993).

The present investigation was carried out to evaluate the role of oxidative metabolism in *Xcc*-infected Buddha Hand (*Citrus medica* var. *sarcodactylis*). Our study revealed that this ornamental plant accumulated higher levels of H_2O_2 after *Xcc* infiltration compared to controls by increasing the activities of SOD and POD and decreasing the activity of CAT.

Materials and Methods

Plant material and culture. Buddha Hand plants were purchased from a commercial nursery (Harris Citrus Nursery, Lithia, FL) and maintained in 15 × 8 cm plastic pots containing Fafard Mix 4P (Southern Agricultural Insecticides Inc., Palmetto, FL). Mineral nutrition was provided periodically using Peters' professional fertilizer (The Scott Co., Marysville, OH). Plants were pruned 90 d before initiation of the experiment and grown in environmental growth chambers. Temperatures ranged from 23 °C (74.4 °F) to 27 °C (80.6 °F) and relative humidity was maintained between 65% and 95%.

Bacterial strain and inoculation. The source of inoculum used in this study was strain *Xac*-NK08 isolated from a foliar le-

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sion from a 'Valencia' tree located in a commercial citrus grove near Immokalee, FL and confirmed using *Xac* Immuno Strips (Agdia Inc., Elkhart, IN). The strain was stored in 10% glycerol at –80 °C. Bacterial cultures were streaked onto nutrient agar (Difco Laboratories, Detroit, MI) prior to use in experiments. For inoculum production, bacteria were transferred from nutrient agar to a flask containing nutrient broth (Difco Laboratories) and grown at 28 °C (82.4 °F) for 24 h in a shaker incubator. Cells were harvested in the late log phase and adjusted to a final concentration of 10^8 cfu/mL using a spectrophotometer (Bio-Spec Mini, Shimadzu Corp., Columbia, MD) at 600 nm. Bacterial concentration was confirmed by counting colonies using serial dilution of the bacterial suspension on nutrient agar after 3 d.

Basal matured leaves (fully expanded) were inoculated using a tuberculin syringe (1 cc) without needle by the method of Viloria et al. (2004). Inoculum was slowly infiltrated into the abaxial leaf surface on both sides of the mid vein to produce a zone of water-soaked tissue 2 mm beyond the diameter of the syringe opening. The water-soaked area varied from 22- to 25-mm diameter and contained about 34–38 µL of *Xac* suspension. Control leaves were mock inoculated with sterile nutrient broth. Five plants were used for each treatment.

Population dynamics. Two leaf discs (7-mm diameter) were excised from the infiltrated region of leaves. Three leaves per plant were removed at every sampling period from three randomly selected plants. Leaf discs were ground in 1 mL of sterile water. Ten-fold serial dilutions of each sample were plated on nutrient agar and incubated at 28 °C (82.4 °F). The colonies were counted after 3 d and used to calculate mean cfu/cm^2 (Lund et al., 1998).

H_2O_2 concentration. Leaf samples (0.5 g) were homogenized in 0.1% trichloroacetic acid and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant (0.3 mL) was mixed with 1.3 mL potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide solution and incubated for 5 min before measuring oxidation product at A_{390}. H_2O_2 concentration was calculated from a standard curve prepared from known concentrations of H_2O_2 and expressed as µmol·g^{-1} FW (Velikova et al., 2000). Positive and negative controls were also assayed.

Leaf preparation for enzyme assays. Leaf samples (0.5 g) were homogenized in 100 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 2 % PVP in a pre-chilled pestle and mortar. The homogenate was centrifuged at 4 °C for 30 min at 15,000 g. For enzymatic analysis the supernatant was gel filtered over Sephadex G-25 (PD-10 column, GE Healthcare Bio-Science AB, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer pH 7.0. All enzyme activities were measured in a final volume of 3 mL using various aliquots of the supernatants (10–25 µL for SOD; 70–125 µL for CAT; 10–50 µL for POD).

Superoxide dismutase. SOD activity was determined by the method of Dhindsa et al. (1981). A preliminary study revealed that overnight dialysis of crude extract using cellulose ester dialysis tubing (MWCO 8000–10000 Dalton) showed no interference by small molecular weight impurities. Similar results were also reported in different plant species such as pea, oat, and corn (Giannopolitis and Ries, 1977). The assay solution (total volume 3.0 mL) contained 201 mM methionine, 1.72 mM NBT, appropriate aliquot of (10–25 µL) enzyme extract, 50 mM potassium phosphate buffer (pH 7.8), and 0.12 mM riboflavin. The riboflavin was added last. The 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 was run for 10 min. The tubes were covered with black cloth immediately after switching off the light. Non-irradiated reaction mixtures containing the enzyme extract, which did not develop color, were used as controls. Blanks lacked enzyme in the reaction mixture and developed maximum color. Absorbance of the reaction mixture was measured at 560 nm. One unit of SOD activity was defined as the enzyme activity that caused a 50% inhibition of the initial rate of the reaction in the absence of enzyme (Dhindsa et al., 1981). Activity was expressed in units of min^{-1} mg^{-1} protein.

Catalase (CAT). CAT activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and leaf extract. The decomposition of H_2O_2 was followed at 240 nm (extinction coefficient = 39.4 mM cm^{-1}; Cakmak and Marschner, 1992). Protein content was determined by the method of Bradford (1976) using BSA as the standard.

Peroxidase (POD). POD 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 leaf extract. 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).

Statistical analysis. 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). Three independent experiments were conducted. Mean values per plant were determined and subjected to analysis of variance (SAS Institute, Cary, NC) with means separated using a protected least significant difference (LSD) at $P < 0.05$. The standard error (SE) of the mean was also calculated.

Results and Discussion

Initial events of canker development following artificial inoculation of *Xcc* in Buddha Hand were similar to other citrus species including water soaking (4 dai) and raised epidermis at 6 dai (Kumar et al., 2011c). However, the only characteristic symptom observed in Buddha Hand was the development of small necrotic areas at 8 dai, which later coalesces into a larger dead necrotic zone (Fig. 1). In addition, the epidermis in Buddha Hand never ruptured and infected leaves remained intact on

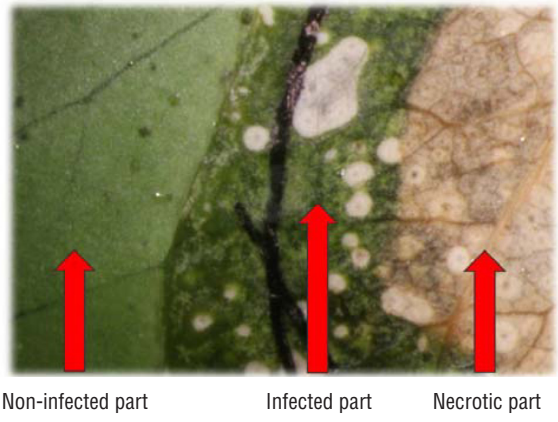

Fig. 1. Microscopic view of epidermis of *Xcc*-infected leaf of Buddha Hand showing non-infected, infected, and necrotic parts at 8 days after inoculation.

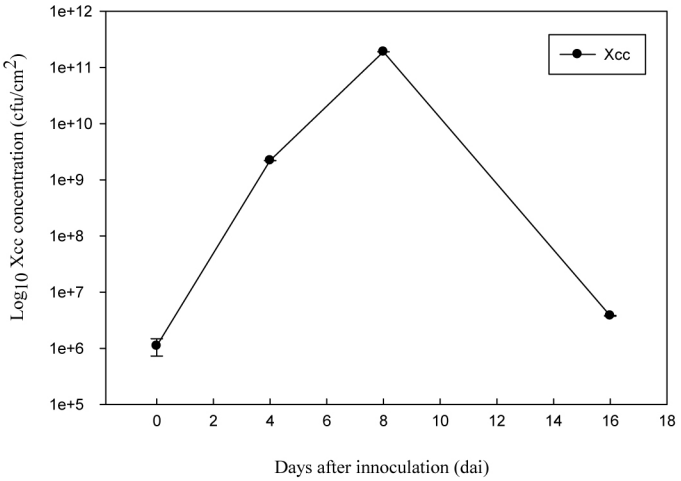

Fig. 2. Bacterial population (cfu/cm^2) at different stages of following inoculation in *Xcc* infiltrated leaves of Buddha Hand. The standard error (± SE) of each mean (n = 15) was calculated and means were consider different when 2 × SE of the mean did not overlap.

Table 1. H_2O_2 concentration and SOD activity in leaves of Buddha Hand at different stages following inoculation of *Xcc*. The standard error (±SE) of each mean (n = 15) was calculated and asterisks indicate these mean values that are significantly different from the controls (means separated using LSD at $P < 0.05$).

	H_2O_2 (μmol g^{-1} fw)		SOD (units mg^{-1} protein min^{-1})	
DAI[z]	CON	*Xcc*	CON	*Xcc*
0	1.9 (± 0.2)	2.0 (± 0.1)	0.9 (± 0.01)	1.0 (± 0.03)
4	2.1 (± 0.4)	2.3 (± 0.3)	1.0 (± 0.02)	1.4 (± 0.02)
8	2.0 (± 0.3)	2.9 (± 0.2)*	0.8 (± 0.01)	1.0 (± 0.01)*
16	2.0 (± 0.3)	7.1 (± 0.8)*	0.9 (± 0.02)	1.5 (± 0.02)*

[z]Days after inoculation.

Table 2. CAT and POD activity in leaves of Buddha Hand at different stages following inoculation of *Xcc*. The standard error (± SE) of each mean (n = 15) was calculated and asterisks indicate these mean values that are significantly different from the controls (means separated using LSD at $P < 0.05$).

	CAT (μmol H_2O_2 mg^{-1} protein min^{-1})		POD (μmol H_2O_2 mg^{-1} protein min^{-1})	
DAI[z]	CON	*Xcc*	CON	*Xcc*
0	0.04 (± 0.001)	0.04 (± 0.001)	1.2 (± 0.2)	1.3 (± 0.1)
4	0.05 (± 0.002)	0.02 (± 0.002)*	0.7 (± 0.2)	0.9 (± 0.1)*
8	0.06 (± 0.001)	0.02 (± 0.001)*	0.5 (± 0.1)	1.1 (± 0.3)*
16	0.05 (± 0.002)	0.07 (± 0.001)*	1.2 (± 0.2)	2.1 (± 0.2)*

[z]Days after inoculation.

plants longer than kumquat, hamlin, and grapefruit (Kumar et al., 2011a, 2011c, 2011d). These distinct features of Buddha Hand are of ecological importance: 1) intact epidermis encased the *Xcc* within the leaf and prevented its infection to surrounding leaves; 2) the delayed abscission of infected leaves limited the dispersal of inoculum; and 3) formation of a necrotic zone in *Xcc*-infected leaves is characteristic of a hypersensitive response that kills the *Xcc* population.

In vivo *Xcc* populations increased from an initial population of 1.1×10^6 cfu/cm^2 (0 dai) to 1.9×10^{11} cfu/cm^2 (8 dai) and then declined by 1000-fold to 3.8×10^8 cfu/cm^2 at 16 dai (Fig. 2). This decline in *Xcc* populations coincided with higher accumulation (7.1 μmol·g^{-1} fw) of H_2O_2 at 16 dai (Table 1). A similar response was observed in kumquat where higher concentrations of H_2O_2 were found to be associated with 75% decline in *Xcc* populations (Kumar et al., 2011a). The levels of H_2O_2 consistently increased in *Xcc*-infected leaves and showed a rise of 60% in-between 8 and 16 dai. H_2O_2 is a reactive molecule and can induce defense-related signaling during pathogen attack (Lamb and Dixon, 1997). H_2O_2 is also antimicrobial and has been shown to inhibit the growth of *Xcc* populations in vitro (Tondo et al., 2010b). The metabolic levels of H_2O_2 in a system are regulated by the rate of its production and destruction by corresponding enzymes (Imlay, 2008). Superoxide dismutase plays an important role in H_2O_2 production during *Xcc*–kumquat interaction (Kumar et al., 2011a). Superoxide dismutase activity in leaves of Buddha Hand was highest (1.5 units mg^{-1} protein min^{-1}) at 16 dai and coincided with the peak production of H_2O_2 (Table 1). However, SOD activity declined at 8 dai but the levels of H_2O_2 remained higher, which indicates that some other sources of H_2O_2 generation were also existing in Buddha Hand.

Catalase removes bulk of H_2O_2 from plant cells (Mittler, 2002) and its activity declined by 60% (4 dai) and 67% at 8 dai, thus promoting the accumulation of H_2O_2 to suppress *Xcc* growth. Interestingly, CAT levels again were enhanced at 16 dai and reached maximum levels (0.07 μmol H_2O_2 mg^{-1} protein min^{-1}) but levels of H_2O_2 never declined. This might have been an *Xcc*-induced response of host machinery enhancing the levels of CAT that consequently lower the concentration of H_2O_2 and facilitate less oxidized preferable environment for *Xcc*. This effect of *Xcc* may have been nullified by maximum activity of POD (2.1 μmol H_2O_2 mg^{-1} protein min^{-1}) at 16 dai to contribute to a H_2O_2 burst (Table 2). *Xcc* only suppressed the POD activity at 4 dai and thereafter, POD activity consistently increased. Class III cell wall peroxidases have been shown to play a critical role in the oxidative burst (Daudi et al., 2012). The hypersensitive response in *Xcc*-infected leaves of Buddha Hand was mediated by higher activities of SOD and POD with simultaneous suppression of CAT activity. Higher accumulation of H_2O_2 and subsequent decline (1000-fold) in *Xcc* populations partly showed the role of oxidative metabolism in *Xcc*–Buddha Hand interaction.

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