Detection of Phenylpropanoids in Citrus Leaves Produced in Response to *Xanthomonas citri* subsp. *citri*

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ABSTRACT

Citrus canker (CC), caused by the bacterial pathogen *Xanthomonas citri* subsp. *citri*, impacts citrus production in many areas of the world by reducing yields, degrading tree health, and severely blemishing the outer peels of fresh fruit. The relative susceptibility to CC among different species of *Citrus* varies from the highly susceptible lime (*Citrus × aurantifolia*), sweet orange (*C. × sinensis*), and grapefruit (*C. × paradisi*) to the much less susceptible calamondin (*C. × microcarpa*) and kumquat (*C. japonica*). This investigation compares the responses to infection with *X. citri* subsp. *citri* of these five genotypes with respect to phenylpropanoid compound profiles and relative increases or decreases of specific compounds postinoculation. In response to *X. citri* subsp. *citri* infection, all hosts possessed increased concentrations of phenylpropanoids in leaf tissue, whereas the similarly treated nonhost orange jessamine (*Murraya*

paniculata) did not. Several of the tested genotypes exhibited notably increased production of fluorescent phenylpropanoids, including umbelliferone, herniarin, auraptene, scoparone, and others. The profiles of these compounds and their levels of production varied among the tested species yet all investigated *Citrus* genotypes exhibited increased concentrations of phenylpropanoids regardless of their degree of susceptibility to *X. citri* subsp. *citri*. Kumquat and calamondin, the tested genotypes least susceptible to *X. citri* subsp. *citri*, also exhibited the highest levels of the dihydrochalcone 3',5'-di-*C*-glucosyl phloretin, the aglycone portion of which, phloretin, is a known antibiotic, although levels of this compound were not affected by inoculation with *X. citri* subsp. *citri*.

Keywords: bacteriology, biochemistry and cell biology

Citrus canker (CC), first identified on herbarium samples collected in the early 1800s, spread with the global expansion of citrus cultivation (Dopson 1964; Luthra and Sattar 1942; Schubert et al. 2001). The bacterial pathogen *Xanthomonas citri* subsp. *citri* responsible for CC is transmitted by wind and rain, and is manifested as a nonsystemic infection of brown, corky, erumpent lesions on citrus peels, stems, and leaves (Bock et al. 2005; Gottwald et al. 2007; Graham et al. 2004). The susceptibility to CC varies among different *Citrus* genotypes within the Rutaceae family and, in this present study, the phytochemical responses of the less susceptible kumquat and calamondin are compared with the highly susceptible lime, sweet orange, and grapefruit.

Previous studies have shown that susceptibility within the genus *Citrus* generally decreases with tissue maturity (Lee 1921). Mature tissues show less field susceptibility due to build-ups of epicuticular wax on leaf and fruit surfaces, and to the development of bark cambium on stems (Graham et al. 1992a). Preexisting lesions on mature fruit show a diminishing ability to generate viable *X. citri* subsp. *citri* as the fruit senesces and the lesions age (Gottwald et al.

2009). Immature fruit, for which stomates are not fully open, also seem to be less susceptible than mature fruit (Graham et al. 1992a,b; Lee 1918, 1921); thus, it appears that the ease of the pathogen's access to leaf mesophyll via open stomates is a leading factor influencing citrus field susceptibility.

However, this observation does not fully explain other differences that occur during the development of this disease, nor does it fully explain the dramatically different in planta bacterial populations that occur in the cankers of different *Citrus* genotypes following direct artificial inoculation (Ference et al. 2019; Wang et al. 2011). Previously, methods used for leaf inoculation resulted in damage which likely induced the formation of defense-related compounds regardless of the presence of inoculum (Treutter 2006). However, with the recent development of an inoculation method that minimizes tissue damage, the induction of chemical responses to plant physical damage is avoided and the study of plant responses to infections is possible (Francis et al. 2011; Gottwald and Graham 1992). Symptom development of CC and in planta populations of *X. citri* subsp. *citri* following this artificial inoculation exhibit a wide range of host responses, from faint chlorosis and small and sparse canker lesions and necrotic tissue to high concentrations of inoculum-producing lesions and massive tissue damage (Ference et al. 2019).

Many *Citrus* genotypes produce numerous different phenylpropanoids in response to stress, disease, and tissue damage (del Rio et al. 2004; Lattanzio et al. 2006; Manthey and Narciso 2013; Treutter 2006). Sweet orange has been shown to accumulate certain flavonoids in leaf tissue after exposure to *Phytophthora citrophthora* (del Rio et al. 2004), as well as highly fluorescent coumarins following *X. citri* subsp. *citri* inoculation (Manthey and Narciso 2013). Citrus varieties such as calamondin and kumquat may also possess phenylpropanoids in their peel which have antimicrobial activity, and it has been proposed that these varieties produce flavonoids in response to *X. citri* subsp. *citri* infection (Chen et al. 2012); however, this has yet to be firmly established and the specific compounds are not yet identified. The purpose of this study was to

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compare the different profiles of phenylpropanoids in methanol extracts of *X. citri* subsp. *citri*-inoculated and water-injected (control) leaves in five *Citrus* spp. along with similarly treated leaves of *Murraya paniculata*, a sixth genotype in the Rutaceae family and a nonhost of *X. citri* subsp. *citri*. Emphasis was placed on the detection of newly produced fluorescent coumarins and psoralens, similar to many previously reported compounds in citrus (Stanley and Vannier 1957; Tatum and Berry 1979). Additional analyses were made of other nonfluorescent phenylpropanoids, mainly flavonoids, in the control and *X. citri* subsp. *citri*-inoculated leaf tissues, some of which have been previously shown to exhibit antimicrobial effects.

MATERIALS AND METHODS

Plants. Six different genotypes of the family Rutaceae were selected as test plants covering a range of relative susceptibility from nonhost to highly susceptible hosts to *X. citri* subsp. *citri*. Among the six genotypes tested, five were in the genus *Citrus*. The six different genotypes included 20 seedlings each of orange jessamine (*M. paniculata* (L.) Jack), calamondin (*Citrus × microcarpa* Mabblerley), Ridge Pineapple sweet orange (*C. × sinensis* (L.) Osbeck.), Duncan grapefruit (*C. × paradisi* Macfad.), Key lime (*C. × aurantifolia* (Christm.) Swingle), and 20 scions of Meiwa kumquat (*C. japonica* Thunb.) grafted on Cleopatra mandarin (*C. resnyi*) rootstock. Each group of plants was divided evenly, with half injected with sterile water and half inoculated with *X. citri* subsp. *citri*. Plants were cultivated in pots in a quarantined greenhouse at the United States Department of Agriculture, Horticultural Research Laboratory in Ft. Pierce, FL, U.S.A. At the time of the experiment, plants were 1 to 2 years old and between 15 and 45 cm in height. Plants were pruned to synchronize flush, and fertigated daily with Peters Professional 20-10-20 General Purpose fertilizer (Everris, Dublin, OH, U.S.A.) dissolved at nitrogen at 325 ppm.

Bacterial strains. *X. citri* subsp. *citri* strain A306 was obtained from the Plant Pathology Department of the University of Florida (originally isolated in Brazil) and was cultured on nutrient agar (Becton, Dickinson and Co., Sparks, MD, U.S.A.) for 24 h at 31 °C. Bacteria were scraped from the surface of the agar plates with a disposable 10-μl inoculating loop and deposited into a bottle containing 50 ml of sterile deionized water. The bacterial suspension was visually measured using the McFarland Equivalence Turbidity Standards (Remel, Lenexa, KS, U.S.A.), adjusted to a final concentration of approximately 3×10^8 CFU ml⁻¹, and subsequently verified by plating.

Inoculum was supplied via a 10-ml sterile syringe inserted into a pulse injection apparatus (Pulse NeedleFree Systems, Inc., Lenexa, KS, U.S.A.) in a manner similar to previous experimentation (Francis et al. 2011), with a modified device and with the following differences in application. The device was set to deliver a 0.1-ml injection volume at 40 psi of compressed CO₂. The pulse injection system's injection aperture was placed in contact with the target leaf's abaxial surface, with a hard rubber backstop held firmly against the adaxial surface directly opposite the injection aperture. Two leaves of similar maturity on each of 10 plants received a single air-pulse injection. Leaves of control plants were injected with only sterile deionized water in an identical manner. One leaf per plant would later be removed at 14 days and the other at 21 days postinoculation (DPI).

Leaf sample preparation. Leaves were detached for tissue collection at 14 and 21 DPI from each of 10 plants per genotype. These two sampling points were based on preliminary experiments which showed little in the way of accumulation of detectable fluorescent compounds in calamondin and grapefruit within the first 2 weeks after inoculation of *X. citri* subsp. *citri* (data not shown). Three discs of leaf tissue were removed from around the inoculation site for each leaf using a stainless steel cork borer (10-mm internal diameter [i.d.]). Each disc contained approximately two parts

symptomless tissue and one part CC symptomatic tissue. Each of the three discs was placed into one of three 25-ml glass vials. Finally, for each genotype or sampling period (14 or 21 DPI), there were three vials, each containing 10 discs of leaf tissue, with 1 leaf disc from each of the 10 different trees within that genotype group. These vials were immediately filled with 10 ml of methanol, sealed, protected from light, and placed on an orbital shaker at 100 rpm for 24 h. After 24 h, the methanol from each vial was removed and placed in a second vial. The original vials with the leaf discs were refilled with another 10 ml of methanol and shaken for an additional 24 h. The two 10-ml methanol samples for each set of leaf discs were combined and were reduced under vacuum to dryness. Methanol (0.40 ml) was added to dissolve the dried solids, then transferred to an autosample vial for high-performance liquid chromatography (HPLC) photodiode array mass spectrometry (PDA-MS) and HPLC-fluorescence analysis.

Compound identification. Compound profiles were analyzed by HPLC-fluorescence detection using a Varian HPLC with ProStar 210 pumps and a ProStar 410 autosampler controlled by Star software (ver. 6.41; Varian, Walnut Creek, CA, U.S.A.). Compound separations were achieved with a Waters XBridge C8 column (4.6 × 150 mm i.d.) (Waters, Milford, MA, U.S.A.). Elution conditions included gradients of aqueous 0.5% formic acid/acetonitrile, initially composed of (90/10, vol/vol), and changed in compositions in linear gradients to 80/20, 75/25, 60/40, 30/70, 30/70, 90/10, and 90/10 (vol/vol) by 10, 15, 23, 40, 45, 53, and 60 min, respectively, at a flow rate of 0.75 ml min⁻¹. Peak detection was achieved with a Jasco FP-2020 fluorescence HPLC detector. Fluorescence peak detection occurred at an emission wavelength of 335 nm, with an excitation wavelength of 400 nm. Fluorescence data were recorded and processed with ChromNAV (Jasco, Tokyo, Japan).

Further compound identification was done through matching molecular weights measured by a Waters ZQ mass spectrometer, and by observing exact peak overlaps with commercial standards. Verification was achieved by matching the fluorescence emission spectra of the leaf compounds with those of commercial standards. Commercial standards included bergamottin (5-geranyloxypsoralen) and herniarin (7-methoxycoumarin) (Indofine Chemical Company, Inc., Hillsborough, NJ, U.S.A.), limettin (5,7-dimethoxycoumarin) (Fluka Analytical, Steinheim, Germany), scopolin (scopoletin 7-*O*-glucoside) (TargetMol, Boston, MA, U.S.A.), scopoletin (6-methoxyumbelliferone) and scoparone (6,7-dimethoxycoumarin) (Aldrich Fine Chemicals, Milwaukee, WI, U.S.A.), and umbelliferone (7-hydroxycoumarin) (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Determination of limits of detection and quantitation. The limit of detection (LoD) and limit of quantitation (LoQ) were determined by calculating the mean instrument response value (microvolts) of 25 samples without analyte at the retention times corresponding to the identified fluorescent compounds, using the following formulas (Aldeek et al. 2015; Armbruster and Pry 2008): LoD = mean_{blank} + 3(SD_{blank}) and LoQ = mean_{blank} + 10(SD_{blank}).

Known concentrations of commercial standards of eight compounds were run using the HPLC, column, fluorescent detector, and elution gradient as described above. LoD and LoQ concentrations (milligrams per liter) of compounds were determined via linear regression equations formulated to resolve the relationship between concentration (milligrams per liter) and fluorescent peak intensity (microvolts) (Table 1).

Statistical analysis. Maximum peak intensity heights from the fluorescence data collected on ChromNAV were input into the JMP statistical suite (version 11.2.0; SAS Institute, Cary, NC, U.S.A.). Data were evaluated using analysis of variance and, subsequently, Tukey's honestly significant difference test. The significance was defined at *P* < 0.05 (Table 2).

RESULTS

Each of the *Citrus* genotypes examined in this study exhibited physical and chemical evidence of disease in the leaves inoculated

with *X. citri* subsp. *citri*. The physical appearances of the canker lesions at 21 DPI are shown in Figure 1, and are similar to those published previously (FERENCE et al. 2019). Leaf surfaces of the less susceptible kumquat and calamondin exhibited lesions with far less total tissue damage compared with the highly susceptible grapefruit, lime, and sweet orange while, for the highly susceptible genotypes, both sides of the leaves showed extensive tissue damage. As anticipated, there were no lesions on the inoculated leaves of orange jessamine, nonhost to *X. citri* subsp. *citri*. Phytochemical

analyses of the five *Citrus* spp. were run to gain further information about the chemical changes that occur in response to *X. citri* subsp. *citri* infection. To conduct these analyses, methanol extracts of *X. citri* subsp. *citri*-inoculated and water-injected leaf tissues of each species were examined by HPLC-UV-MS as well as by HPLC-fluorescence detection, which provides highly sensitive and selective detections of fluorescent coumarins and psoralens.

Calamondin. The HPLC-fluorescence chromatograms of extracts of *X. citri* subsp. *citri*-inoculated calamondin leaves

TABLE 1. Limit of detection (LoD), limit of quantitation (LoQ), and linear relation of eight fluorescent compounds

Compound	LoD (mg/liter)	LoQ (mg/liter)	Regression equation (concentration–peak area)	Correlation coefficient
Tryptophan	837.80	4,364.98	$y = 0.13x + 140.32$	0.9088
Scopoletin	0.05	0.08	$y = 11342.37x - 308.33$	1
Umbelliferone	0.15	0.49	$y = 3820.47x + 182.78$	1
Scoparone	0.11	0.31	$y = 21452.65x - 257.22$	1
Herniarin	0.10	0.19	$y = 32126.81x - 1953.61$	0.9998
Limettin	0.02	0.05	$y = 30329.41x - 46.56$	1
Auraptene	0.03	0.05	$y = 9761.88x + 826.83$	0.9998
Bergamottin	24.99	132.99	$y = 6.74x + 285.61$	0.9976

TABLE 2. Mean values of fluorescence intensity units of fluorescent compounds in *Xanthomonas citri* subsp. *citri*-inoculated and control leaves from five different *Citrus* plants^z

Compounds, sample group	Fluorescence intensity units (μV) per species				
	Calamondin	Grapefruit	Kumquat	Lime	Sweet orange
Tryptophan					
Control	ND B, c	1,205 B, b	430 B, c	2,254 B, a	289 B, c
14 DPI	4,989 A, c	22,131 A, a	7,322 A, bc	11,253 A, b	5,223 A, c
21 DPI	5,050 A, b	30,734 A, a	4,883 AB, b	11,117 A, b	6,838 A, b
Scopoletin					
Control	ND B, b	401 C, b	942 B, a	1,177 B, a	259 C, b
14 DPI	1,658 A, b	2,886 B, b	1,459 AB, b	14,470 A, a	2,567 B, b
21 DPI	1,949 A, b	4,605 A, b	1,690 A, b	14,544 A, a	3,972 A, b
Umbelliferone					
Control	ND B, b	ND C, b	ND A, b	3,247 B, a	ND C, b
14 DPI	826 B, b	4,028 B, a	ND A, b	4,048 A, a	811 B, b
21 DPI	2,362 A, bc	13,465 A, a	ND A, c	4,707 A, b	1,458 A, c
17.5 min unknown					
Control	ND C, c	ND C, bc	ND B, ab	ND C, a	ND C, c
14 DPI	4,940 B, b	2,117 B, b	2,070 B, b	20,386 B, a	5,813 B, b
21 DPI	9,797 A, bc	4,397 A, c	2,495 A, c	35,004 A, a	17,042 A, b
Scoparone					
Control	ND C, a	ND C, a	ND B, a	ND C, a	ND C, a
14 DPI	12,826 B, b	ND B, c	2,223 AB, c	26,984 B, a	13,867 B, b
21 DPI	25,270 A, b	3,638 A, c	2,929 A, c	46,679 A, a	45,610 A, a
Herniarin					
Control	ND B, b	1,948 A, b	ND A, b	751,003 A, a	ND B, b
14 DPI	4,959 B, b	1,998 A, b	2,936 A, b	598,347 B, a	1,964 A, b
21 DPI	31,946 A, b	2,477 A, b	2,815 A, b	490,805 B, a	2,710 A, b
Limettin					
Control	ND B, b	506 C, b	ND A, b	154,271 A, a	ND B, b
14 DPI	645 A, b	1,929 B, b	517 A, b	151,085 A, a	4,242 A, b
21 DPI	665 A, b	2,448 A, b	484 A, b	162,810 A, a	4,363 A, b
29.5 min unknown					
Control	ND B, a	ND B, a	ND B, a	ND B, a	ND B, a
14 DPI	15,412 A, b	25,247 A, ab	1,237 A, c	24,713 A, ab	35,366 A, a
21 DPI	16,660 A, bc	32,072 A, b	1,326 A, c	26,865 A, b	52,460 A, a
Auraptene					
Control	ND B, a	ND B, a	693 B, a	619 A, a	644 A, a
14 DPI	4,006 B, a	692 A, b	998 A, b	701 A, b	768 A, b
21 DPI	8,516 A, a	724 A, b	924 AB, b	729 A, b	870 A, b
Bergamottin					
Control	1,002 A, b	1,219 A, b	1,069 A, b	95,622 A, a	1,408 A, b
14 DPI	888 A, b	1,218 A, b	1,069 A, b	90,246 A, a	1,018 A, b
21 DPI	887 A, b	1,092 A, b	1,012 A, b	89,079 A, a	964 A, b

^z For the same compound, mean values followed by different uppercase letters within a column and different lowercase letters within a row indicate significant differences using Tukey's honestly significant difference test ($P < 0.05$); ND = value below limit of detection. *Citrus* plants: Calamondin (*Citrus × microcarpa*), Duncan grapefruit (*C. × paradise*), kumquat (*C. japonica*), Key lime (*C. × aurantifolia*), and Ridge Pineapple sweet orange (*C. × sinensis*). DPI = days postinoculation.

exhibited multiple fluorescent peaks not present, or present at only trace amounts, in the water-injected leaves (Table 2; Fig. 2). In these chromatograms, major peaks occurred at 18.5 min for scoparone, 23.0 min for herniarin (7-methoxycoumarin), 39.5 min for auraptene (7-geranyloxycoumarin), and two unknown compounds at 17.5 and 29.5 min (Fig. 2). Smaller peaks appeared in the extracts of the *X. citri* subsp. *citri*-inoculated calamondin leaves for tryptophan, scopolin (scopoletin-7-*O*-glucoside), scopoletin, and umbelliferone at 6.1, 6.7, 11.5, and 14.0 min, respectively. In sharp contrast, umbelliferone, tryptophan, herniarin, auraptene, scoparone, and the unknowns occurred below the LoD in control samples (Table 2). The fluorescent peak intensities for tryptophan, scopoletin, umbelliferone, scoparone, and the 17.5- and 29.5-min unknowns were significantly higher in *X. citri* subsp. *citri*-inoculated leaves at 14 DPI than in water-injected controls (Table 2; Fig. 2). Herniarin and auraptene were significantly higher in *X. citri* subsp. *citri*-inoculated leaves at 21 DPI than in water-injected controls (Table 2; Fig. 2). Umbelliferone, scoparone, herniarin, and auraptene peaks increased in intensity in the *X. citri* subsp. *citri*-inoculated leaves during the period between 14 and 21 DPI (Table 2; Fig. 2).

Analysis of calamondin leaf extracts showed the occurrence of a large number of minor-occurring flavone glycosides as well as phloretin-di-*C*-glucoside at 13.2 min, all of which were unaffected by inoculation with *X. citri* subsp. *citri* (Fig. 3). Sinensetin, nobiletin, tetramethoxyscutellarein, and tangeretin were detected, as well as other minor polymethoxylated flavones (PMFs) (data not

shown). In addition to these compounds, there were also three fluorescent lipophilic unknown compounds with similarly unique UV spectra (Fig. 4). These compounds were detected only in the calamondin leaf extracts and showed no differences in amounts between water-injected control and *X. citri* subsp. *citri*-inoculated samples and, thus, did not appear to be part of a response of calamondin to *X. citri* subsp. *citri* infection.

Kumquat. Extracts of *X. citri* subsp. *citri*-inoculated kumquat leaves produced low-intensity peaks (compared with the newly formed peaks in the other citrus varieties) at 6.8, 6.9, 11.5, 17.5, 18.5, 29.5, and 39.5 min (Fig. 5A). In *X. citri* subsp. *citri*-inoculated kumquat leaves, tryptophan (6.8 min), scopolin (6.9 min), scopoletin (11.5 min), scoparone (18.5), auraptene (39.5 min), and the two unknown compounds similarly detected in calamondin at 17.5 and 29.5 min were all induced by *X. citri* subsp. *citri* inoculation (Table 2). In the control leaves, the peaks due to herniarin, limettin, scoparone, and the unknowns at 17.5 and 29.5 min were below the LoD values for these compounds. Tryptophan, auraptene, and the unknown at 29.5 min were significantly higher in *X. citri* subsp. *citri*-inoculated leaves at 14 DPI than in water-injected controls (Table 2). Scopoletin, scoparone, and the unknown at 17.5 min significantly increased in concentration during the period from 14 to 21 DPI, and were significantly higher in *X. citri* subsp. *citri*-inoculated leaves at 21 DPI than in control leaves (Table 2). In the water-injected and *X. citri* subsp. *citri*-inoculated leaves, very similar profiles of phloretin 3',5'-di-*C*-glucoside (13.2 min), isomargaretin (14.9 min), sinapinic acid glucoside (15.1 min),

Fig. 1. Physical appearances of the canker lesions at 21 days postinoculation on leaves of the five *Citrus* hosts and the nonhost orange jessamine.

Fig. 2. Typical high-performance liquid chromatography chromatogram of methanol extracts of calamondin leaf tissue from A, control at 21 days postinoculation (DPI) and *Xanthomonas citri* subsp. *citri*-inoculated samples at B, 14 DPI and C, 21 DPI. Letters T, S1, S2, S3, U, H, Uk, L, B, and A represent fluorescent peaks due to the presence of tryptophan, scopolin, scopoletin, scoparone, umbelliferone, herniarin, unknown compounds at 17.5 and 29.5 min, limettin, bergamottin, and auraptene, respectively.

fortunellin-glucoside (16.2 min), margaretin (17.2 min), and fortunellin (20.2 min) were observed (data not shown).

Grapefruit. Extracts of *X. citri* subsp. *citri*-inoculated grapefruit leaves showed intense fluorescence peaks at 6.9, 11.5, 13.5, 17.5, 18.5, 27.5, 29.5, 32.3, 35.5, 39.5, 45.0, and 44.8 min at 14 and 21 DPI (Table 2; Fig. 5B). The identified peaks represent tryptophan/scopolin (overlapping) (6.9 min), scopoletin (11.5 min), umbelliferone (13.5 min), scoparone (18.5 min), limettin

(27.5 min), auraptene (39.5 min), and the two unknowns observed in calamondin at 17.5 and 29.5 min. Peaks due to umbelliferone, auraptene, scoparone, and the unknowns at 17.5 and 29.5 min were below the LoD in control samples. Tryptophan, scopoletin, umbelliferone, scoparone, limettin, auraptene, and the two unknowns at 17.5 and 29.5 min were significantly higher in *X. citri* subsp. *citri*-inoculated leaves at 14 DPI than in water-injected controls (Table 2). Fluorescent peaks due to umbelliferone,

Fig. 3. High-performance liquid chromatography chromatogram of calamondin leaf extracts. Peaks with retention times of 7.91, 8.28, 9.95, 11.05, 11.58, and 12.51 min exhibited mass spectra indicative of flavone-C-glycosides. The peak at 13.21 min was identified as 3',5'-di-C-glucosylphloretin. Peaks at 15.48 (diosmin), 16.15, and 17.11 (neohesperidin) min exhibited mass spectra indicative of flavonoid-O-glycosides.

scopoletin, scoparone, limettin, and the unknown at 17.5 min further increased in intensity in the *X. citri* subsp. *citri*-inoculated leaves during the period between 14 and 21 DPI (Table 2). HPLC-MS analysis of the leaf extracts showed low concentrations of the PMFs sinensetin, nobiletin (26.9 min) (Fig. 5B), tetramethylscutellarein, and tangeretin, (data not shown). The same concentrations of these compounds occurred in the *X. citri* subsp. *citri*-inoculated and the control water-injected leaves. Large fluorescent peaks at retention times 26.9 min (nobiletin), 32.4 (unknown), 36.0 min (6',7'-epoxybergamottin), and 45.0 min (unknown) occurred regardless of the inoculation treatment. Additional analyses of the flavonoids in grapefruit leaves showed evidence of naringin, rhoifolin, and other flavone glycosides, as reported previously (Manthey et al. 2000).

Lime. Extracts of *X. citri* subsp. *citri*-inoculated lime leaves at 21 DPI exhibited intense fluorescent peaks at 6.1 min (tryptophan), 11.5 min (scopoletin), 17.5 min (unknown), 18.5 min (scoparone), 23.0 min (herniarin), 27.4 min (limettin), 29.5 min (unknown), 42.0 min (unknown), and 47.0 min (bergamottin) (Table 2; Fig. 5C). Although the peak associated with herniarin occurred in both water-injected control and *X. citri* subsp. *citri*-inoculated lime leaves, this peak was 26 to 53% higher in the control leaves (Table 2). The other substantial fluorescent peaks in the lime leaf extracts were identified as limettin (27.4 min) and bergamottin (47.0 min) (Fig. 5C). The presence and relative intensities of the limettin and bergamottin fluorescence peaks were independent of *X. citri* subsp. *citri* infection (Table 2). The peaks at 17.5 and 29.5 min were similar to the unknown compounds detected in *X. citri* subsp.

Fig. 4. UV spectra from the high-performance liquid chromatography chromatogram of one of the N-containing unknown compounds in calamondin leaves. Two other compounds with similar UV spectra also occur in these leaf extracts.

citri-inoculated leaves from all other tested citrus genotypes. Peaks due to scoparone and the unknown at 29.5 min were below the LoD in control samples. Tryptophan, scopoletin, umbelliferone, scoparone, and the two unknowns at 17.5 and 29.5 min were significantly higher in 14 DPI *X. citri* subsp. *citri*-inoculated leaves than in water-injected controls (Table 2). Scoparone and the unknown at 17.5 min further increased in intensity in the *X. citri* subsp. *citri*-inoculated leaves during the period between 14 and 21 DPI (Table 2). Herniarin was significantly lower in *X. citri* subsp. *citri*-inoculated leaves at 14 and 21 DPI than in the control leaves (Table 2). From 14 to 21 DPI, the average peak intensity in the extracts of *X. citri* subsp. *citri*-inoculated leaves associated with scoparone showed a 58% increase, whereas those for tryptophan, scopolin, scopoletin, herniarin, umbelliferone, limettin, bergamottin, and the unknown compound detected at 29.5 min were unchanged (Table 2).

The chemical composition of the lime leaf extracts were further analyzed with HPLC-PDA-MS. These analyses showed numerous coumarins and psoralens. Compounds identified by UV and MS analysis included, in part, tryptophan, vicinen-2, diosmin, hesperidin, *p*-coumaric acid, herniarin, angelicin, bergapten, limettin, isopimpinellin, xanthotoxol geranyloxy ester, and bergamottin. Based on the observations made with the control and *X. citri* subsp. *citri*-inoculated leaf samples, very little change occurred in the levels of these phenylpropanoids in response to infection with *X. citri* subsp. *citri*.

Sweet orange. HPLC chromatograms of the extracts of sweet orange leaves inoculated with *X. citri* subsp. *citri* showed fluorescent peaks at 6.1, 6.7, 11.5, 17.5, 18.5, 21.0, 23.5, 29.5, 32.5, 35.5, 38.5, and 40.1 min. Major peaks identified were 6.1 (tryptophan), 6.7 (scopolin), 11.5 (scopoletin), 17.5 (unknown), 18.5 (scoparone), and 29.5 (unknown) min (Fig. 5D). Minor peaks at 14.0 (umbelliferone), 22.5 (herniarin), and 27.4 (limettin) min were also detected. Peaks due to umbelliferone, herniarin, limettin, scoparone, and the unknowns at 17.5 and 29.5 min were below the LoD in control samples. Tryptophan, scopoletin, umbelliferone, scoparone, herniarin, limettin, and the two unknowns at 17.5 and 29.5 were significantly higher in *X. citri* subsp. *citri*-inoculated leaves at 14 DPI than in water-injected controls (Table 2). Scopoletin, umbelliferone, and scoparone further increased in intensity in the *X. citri* subsp. *citri*-inoculated leaves during the period between 14 and 21 DPI (Table 2). An intense fluorescent peak for an unknown compound at 32.5 min was higher in control samples (data not shown).

Orange jessamine. No new production of fluorescent phenylpropanoids, as seen in other genotypes, occurred after *X. citri* subsp. *citri* inoculation. Additionally, the HPLC chromatograms monitored at 335 and 400 nm exhibited no differences between the control and *X. citri* subsp. *citri*-inoculated leaf extracts. Fluorescent peaks from inoculated and noninoculated orange jessamine leaf tissues showed no significant differences between the two groups

Fig. 5. High-performance liquid chromatography chromatograms of methanol extracts of **A**, Meiwa kumquat; **B**, Duncan grapefruit; **C**, Key lime; and **D**, Ridge Pineapple sweet orange leaf tissue at 21 days postinoculation. Letters A, B, eB, H, L, N, S1, S2, S3, T, U, and Uk represent fluorescent peaks due to the presence of auraptene, bergamottin, epoxybergamottin, herniarin, limettin, nobiletin, scopolin, scopoletin, scoparone, tryptophan, umbelliferone, and unknown compounds at 17.5 and 29.5 min, respectively.

(control and *X. citri* subsp. *citri*) or between the two sampling dates (14 and 21 DPI). Orange jessamine leaf tissue exhibited a number of intense fluorescent peaks at 2.9, 19.0, 21.0, 22.0, 28.4, 31.0, 37.5, 43.0, and 45.5 min, none of which corresponded to the intense fluorescent peaks noted in the other investigated genotypes (Table 2). A concurrent HPLC-UV analysis of the orange jessamine leaf extracts showed a preponderance of coumarins and early-eluting hydroxycinnamates and a nearly complete absence of flavonoids. The fluorescent peaks at 19.0, 22.0, 28.4, 31.0, and 37.5 min exhibited coumarin-like UV spectra. The majority of coumarins thus far identified in orange jessamine are constructs of 5,7-dihydroxycoumarin with different isopentenyl-based substituents positioned at the coumarin C8 position (Aziz et al. 2010; Imai et al. 1989; Wu 1988). HPLC-MS of the above fluorescent peaks showed prominent 189 *m/z* fragment ions consistent with the occurrence of the above-published 5,7-dihydroxycoumarin adducts (data not shown).

Compounds. Among extracts of water-injected control leaves, fluorescent peaks due to tryptophan, scopoletin, umbelliferone, herniarin, limettin, and bergamottin were all highest in lime samples (Table 2). Scoparone and the unknowns at 17.5 and 29.5 were below the LoD in the control leaves of all genotypes, including lime (Table 2). For *X. citri* subsp. *citri*-inoculated leaves at 14 and 21 DPI, fluorescent peaks due to scopoletin, umbelliferone, scoparone, herniarin, limettin, bergamottin, and the unknowns at 17.5 and 29.5 min were also highest among lime samples (Table 2).

Flourescent peaks due to umbelliferone, scoparone, herniarin, limettin, auraptene, and the unknowns at 17.5 and 29.5 min were below the LoD in water-injected control leaves from kumquat (Table 2). With the addition of tryptophan and scopoletin, these same compounds were all also below the LoD in water-injected leaves of calamondin (Table 2). Among control leaves, all genotypes had statistically similar values for peaks due to auraptene, with the values from both calamondin and kumquat being below the LoD (Table 2). However, the most intense fluorescent peak due to auraptene was seen in *X. citri* subsp. *citri*-inoculated calamondin samples (Table 2).

DISCUSSION

Our results show that, with the exception of orange jessamine, all tested Rutaceae family genotypes accumulated phenylpropanoids in leaf tissue in response to inoculation with *X. citri* subsp. *citri*, and did so regardless of that specific genotype's relative susceptibility to CC (Table 2). Although orange jessamine, a member of the Rutaceae family, is a nonhost of *X. citri* subsp. *citri*, bacterial populations persist in its leaf tissue for more than 30 days after artificial inoculation (FERENCE et al. 2019). However, the fluorescent peaks produced by orange jessamine leaf tissue samples exhibited no differences between noninoculated and inoculated samples at either 14 or 21 DPI. The fluorescent peaks that are detected in orange jessamine samples are dissimilar to the fluorescent peaks from compounds found in *X. citri* subsp. *citri*-inoculated samples from the other investigated host genotypes. The absence of new production of phenylpropanoids in orange jessamine leaf tissue after inoculation with *X. citri* subsp. *citri*, combined with the absence of correlation between the chromatograms of *X. citri* subsp. *citri*-inoculated orange jessamine samples and of all other *X. citri* subsp. *citri*-inoculated genotypes (calamondin, kumquat, grapefruit, sweet orange, and lime), suggests that fluorescent peaks due to phenylpropanoids found in extracts of *X. citri* subsp. *citri*-inoculated *Citrus* leaves result from interactions between the bacterium and CC-susceptible hosts.

Phenylpropanoids produced by plants have many functions, and the involvement of phenylpropanoids and their derivatives in influencing plant-microbe interactions has been widely studied (Cueva et al. 2010; Daglia 2012; del Rio et al. 2004; Jagani et al. 2009; Joshi et al. 2015; Rauha et al. 2000). Plant phenolics can act as

signals to attract beneficial or protect against harmful microorganisms (Treutter 2006). Some phenylpropanoids provide protection to the plant in adverse environmental conditions or after tissue damage, while others are defensive compounds which are either preformed or induced in response to injury, stress, or infection by pathogens (Treutter 2006). Studies on citrus report that increased tolerance to CC corresponded to higher total phenolic compound concentration in leaves (Jiao et al. 1992). PMFs have previously been found to occur in calamondin, and levels of PMFs and flavanones fluctuate as part of the defense response of Valencia sweet orange to *P. citrophthora* infection (del Rio et al. 2004; Gonçalves et al. 2018; Lou and Ho 2017; Tatum and Berry 1979). Total ethanol extracts from calamondin and kumquat peel, which contain high levels of the flavonoids diosmin and quercetin, were shown to inhibit *X. citri* subsp. *citri* growth in vitro (Chen et al. 2012). Numerous instances of in planta phenylpropanoid gene induction in response to tissue injury and infections have been reported in citrus (Chen et al. 2012; Ference 2016; Ortuño et al. 1997, 2011). Specifically, genes associated with phenylpropanoid biosynthesis and metabolic processes are significantly upregulated in kumquat, calamondin, and sweet orange after exposure to *X. citri* subsp. *citri* (Ference 2016; Fu et al. 2012; Khalaf et al. 2007, 2011).

The production of secondary metabolites, including phenylpropanoids, is a known and widely investigated phenomenon associated with the physiological response of citrus plants to biotic stress, and certain phenylpropanoids found in the tissues of citrus plants are known to be antimicrobial, including being inhibitory specifically toward *X. citri* subsp. *citri*. In alignment with this, *Citrus* plants in our study presented significantly altered chromatograms after inoculation with *X. citri* subsp. *citri*, indicative of the production and accumulation of specific phenylpropanoids in response to biotic stress.

The phloretin-di-C-glucoside found in calamondin leaf tissue is a 3',5'-di-C-glucosyl phloretin and occurs at high concentrations in both calamondin and kumquat (Barreca et al. 2011; Lou and Ho 2017; Yu et al. 2013), the aglycone of which is the antibiotic phloretin (Barreca et al. 2014). The phloretin 3',5'-di-C-glucoside has been shown to occur at levels between 21.3 and 60.2 mg/g dry weight in the leaves of different kumquat varieties (Ogawa et al. 2001).

Calamondin is considered to be only mildly susceptible to *X. citri* subsp. *citri* and, in this study, it was also shown to produce a number of *X. citri* subsp. *citri* infection-induced fluorescent compounds, many of which are also detected in some of the other examined *Citrus* genotypes. A presumption could be made that these compounds are responsible for the low susceptibility of calamondin to *X. citri* subsp. *citri*. However, it is difficult to assign the responsibility of imparting the reduced susceptibility of calamondin to CC to any single example among these several induced compounds. For example, an intense fluorescent peak identified as scoparone appeared in *X. citri* subsp. *citri*-inoculated calamondin leaf tissue but no similarly intense peak was seen in control calamondin tissue (Table 2). The increased production of scoparone in related *Citrus* genotypes in response to the presence of other pathogens has been previously suggested to be a part of a general defense response in calamondin (Ortuño et al. 1997). However, a similar peak for scoparone also occurs in extracts of the *X. citri* subsp. *citri*-infected leaves of the highly susceptible lime, as well as in the *X. citri* subsp. *citri*-susceptible sweet orange, but not in the leaf extracts of *X. citri* subsp. *citri*-infected kumquat, which is equally or more tolerant than calamondin (Ference et al. 2019).

The fluorescent peak at 23.0 min (identified as herniarin) in *X. citri* subsp. *citri*-inoculated calamondin but not found in water-injected calamondin samples occurred at very high intensities in both inoculated and noninoculated lime. Interestingly, this peak was higher in the water-injected than in the *X. citri* subsp. *citri*-inoculated lime leaf extracts. Although the production of herniarin in calamondin seems to be induced by exposure to *X. citri* subsp.

citri, its biosynthesis may be either directly inhibited by the pathogen in the more susceptible lime species or is metabolized after exposure to *X. citri* subsp. *citri*, or its accumulation is lost by the rapid destruction of tissue as a result of canker development, which is more severe in lime than in calamondin (Fig. 1). The constant concentration of limettin in both inoculated and non-inoculated lime tissue suggests against the reduction of herniarin being due to the extensive destruction of tissue. *XacPNP*, a gene of *X. citri* subsp. *citri* encoding a plant natriuretic peptide-like protein, is capable of modifying host metabolism and is involved in the reduction of secondary metabolite production in infected citrus tissue as cellular resources are diverted down other pathways (Gottig et al. 2008). The influence of *X. citri* subsp. *citri* in lime tissue, possibly by means of *XacPNP*, may be involved in the reduced concentration of herniarin. Increased levels of herniarin were also detected in sweet orange at 21 DPI but with a mean peak intensity less than 9% of the mean peak intensity from calamondin at 21 DPI and less than 0.6% of the mean peak intensity from lime at 21 DPI (Table 2). Although 21-DPI extracts from the more tolerant calamondin produced fluorescent peaks due to herniarin which were, on average, an order of magnitude greater than those produced by the more susceptible sweet orange, the herniarin fluorescent peaks from lime at 21 DPI were, on average, an order of magnitude greater than those produced by calamondin; thus, it is again difficult to unambiguously declare, based on our results, a direct role for this compound in inhibiting the growth of *X. citri* subsp. *citri* in the leaf tissue of less susceptible citrus. Among water-injected controls, the majority of the identified compounds, as well as the unknown at 17.5 min, were found to be most abundant in lime relative to the other *Citrus* genotypes. Among *X. citri* subsp. *citri*-inoculated samples at 21 DPI, again, most of the identified compounds were most abundant in lime, with the exceptions of tryptophan and auraptene, as well as the unidentified compound at 29.5 min. Tryptophan was found most abundantly in grapefruit, while the unknown compound at 29.5 min was found to be most abundant in sweet orange at 21 DPI. Levels of umbelliferone were similar in *X. citri* subsp. *citri*-inoculated lime and grapefruit, while levels of scoparone were similar in lime and sweet orange at 21 DPI. However, the difference in susceptibility to *X. citri* subsp. *citri* A306 between sweet orange, grapefruit, and lime is negligible (FERENCE et al. 2019). Auraptene, a coumarin known to reduce the *Helicobacter pylori* colonization of stomach lesions in gerbils, was highest in calamondin among the *X. citri* subsp. *citri*-inoculated group at 21 DPI (Takeda et al. 2007).

Though it is difficult to assign an inhibitory effect to any particular compound, our results also cannot definitively rule out a synergistic growth-inhibitory effect of multiple sets of these compounds in the different *Citrus* genotypes. However, it is important to note that, although many of these coumarins have been shown to exhibit antibacterial properties, the potencies of these compounds are extremely low, as shown from their typically very high minimum inhibitory concentration values (de Souza et al. 2005; Kayser and Kolodziej 1999; Ng et al. 1996).

Although the results of this study did not point out to us any newly formed compounds following *X. citri* subsp. *citri* inoculation that we could unambiguously attribute to *Citrus* genotype-specific susceptibility differences because the production of phenylpropanoids, in either character or quantity, did not directly correlate to susceptibility to *X. citri* subsp. *citri*, it remains perfectly plausible that there might still be undetected induced or constitutively expressed compounds able to limit the growth and virulence of the *X. citri* subsp. *citri* bacteria in leaves of less susceptible hosts. Antimicrobial properties of the phenylpropanoids in citrus leaves have been reported but, in general, the in vitro antibacterial strengths of these compounds are weak. Although this low antibacterial activity is also true for most of the coumarins and psoralens, in some instances these and similar compounds exert strong activities against different bacterial and fungal pathogens

(Céspedes et al. 2006; Yang et al. 2016). In unpublished in vitro trials, we have not observed any potent anti *X. citri* subsp. *citri* activity by the phenylpropanoids reported in Table 2 (data not shown). It is interesting however, that the two least susceptible genotypes, kumquat and calamondin, exhibited the highest levels of the dihydrochalcone 3',5'-di-*C*-glucosyl phloretin, for which the aglycone portion of this compound, phloretin, exhibits powerful antibiotic actions in vitro. However, this compound did not respond to inoculation with *X. citri* subsp. *citri* (Barreca et al. 2014). Other similar dihydrochalcones also exhibit antibacterial effects (Awouafack et al. 2008; Hufford and Oguntimein 1980; Orjala et al. 1994). Although the various glucoside derivatives of phloretin have negligible antibacterial strength, microbial or endogenous plant deglycosidases may liberate the active aglycone at the point of contact with *X. citri* subsp. *citri*. However, whether there are any influences of *X. citri* subsp. *citri* infection on the biosynthesis of the aglycone and its glucosides remains unknown and additional detailed studies of *Citrus* leaf compounds and their changes following *X. citri* subsp. *citri* inoculation are needed.

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