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Detection of Fluorescent Compounds in Citrus Leaf Cankers

JOHN A. MANTHEY* AND JAN A. NARCISO

U.S. Horticultural Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Ft. Pierce, FL 34945

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Citrus canker caused by the pathogenic bacterium, *Xanthomonas citri* subsp. *citri* (*Xcc*) , poses a serious threat to citrus production in Florida, especially for the fresh fruit market. *Xcc* causes severe damage to fruit, stem, and leaf tissues, and although much has been learned about the complex interactions between the infecting bacteria and these citrus plant tissues, chemical responses in host trees to this infection has yet to be fully explored. To address this issue, initial investigations were made of the changes in the phytochemical compositions of canker-infected leaves of greenhouse-grown grapefruit trees. These changes were monitored by HPLC-fluorescence spectroscopy, where particular attention was given to the detection of coumarins (C) and furanocoumarins (FC). Such analyses showed that physical abrasion of grapefruit leaves dramatically decreased the levels of certain Cs and FCs, and that subsequent *Xcc* infections triggered the production of other Cs and FCs.

Citrus canker remains a very serious threat to the Florida citrus industry, particularly to grapefruit production for the fresh fruit market. Citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *citri* (*Xcc*), is typically spread by wind and rain and enters into the stomates and into sites of tissue damage of leaves, fruits, and stems. This is one reason why there was such a dramatic spread of this disease in Florida following the hurricanes in the mid 2000s. Cankers on the peels and leaves of grapefruit are shown in Figure 1. In addition to the unsightly appearance of the peels of such infected fruit, advanced stages of this disease also cause premature fruit drop and early tree failure (Gotwald et al., 2002).

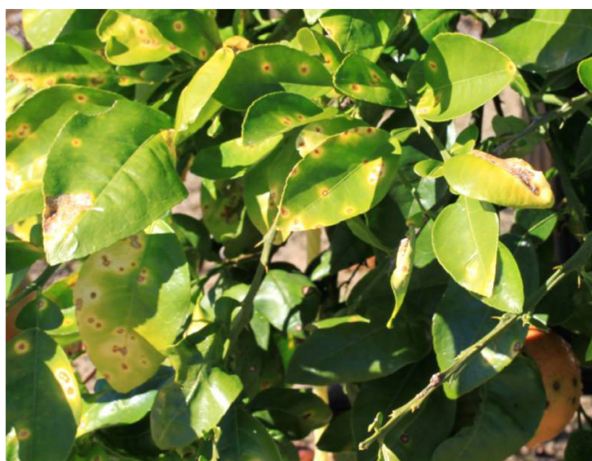

Fig. 1.Visual symptoms of *Xcc* infection on grapefruit peel and leaves.

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*Corresponding author; phone: (863) 293-4133; email: John.Manthey@ars.usda.gov

Although the complex interactions between *Xcc* and the host grapefruit tissues have been widely studied, much still remains to be learned about the chemical responses that occur in these plant tissues following bacterial infection. A certain amount of information about these chemical responses may be inferred by what is already known about the responses in grapefruit tissues to other microbial pathogens. Attack by the green mold (*Penicillium digitatum*) triggers the deposition of phloroglucinol-HCl reactive materials composed of complex mixtures of lignin-like substances and of benzenoid-aldehyde-infused water insoluble carbohydrates (Baudoin and Eckert,1985; Brown and Barmore,1983). These latter materials were lately described as suberin (Lai et al., 2003), and similar depositions in injured leaf tissue were previously linked to increased resistance to infection by *Xcc* in other citrus varieties (Koizumi, 1983). Other chemical changes include increased production of antifungal coumarins (Afek et al., 1986, 1999; Ortuño et al., 2011) and phenolic aldehydes (Stange et al., 1993) (described as putative phytoalexins (D'hallewin et al., 2000). The phytochemical responses in grapefruit to bacterial attack, specifically to *Xcc*, are not as well known, and thus, information concerning how grapefruit controls the growth and virulence of *Xcc* at the site of attack is largely lacking. To begin to characterize such putative phytochemical responses in grapefruit to *Xcc* infection, profiles of fluorescent compounds in healthy and canker-infected leaves were analyzed by HPLC-fluorescence peak detection.

Materials and Methods

INOCULATION. Sixty 2-year-old potted 'Duncan' grapefruit trees were taken from the greenhouse to the laboratory for *Xcc* inoculation studies. Treatments for these studies included wounded but not inoculated leaves, wounded and inoculated with *Xcc*, and healthy, unwounded leaves. Two leaves per treatment were used on each tree (120 leaves per treatment). To create a wound site, leaves of median age were randomly chosen and a small area was wounded with fine, low grit sandpaper. Wounds were made on

the outer edge of each leaf on both the abaxial and adaxial sides. Immediately after wounding, either 5 µL of sterile water, or 5 µL of a suspension of 10^7 *Xcc* cells, were placed on the injury and allowed to dry. Upon drying, small, lightly perforated sleeves made with glassine envelopes were placed over each leaf (i.e., wounded no *Xcc*, wounded with *Xcc*, and no wound) to maintain moisture until wounds began to heal. Plants were placed back in the greenhouse, subjected to normal daylight regime and irrigated once daily by drip method. Sleeves were removed after 1 week and leaves were harvested for extraction when leaf symptoms showed signs of *Xcc* infection, after approximately 4 weeks.

Sample extraction. Leaves were sampled for the chemical compositions of healthy, wounded, and *Xcc*-infected leaves. Leaf segments (20) were cut with a 8-mm-diameter cork borer in healthy non-abraded, non-infected leaves (labeled H), then from injured, but not infected leaves (labeled C), and then from cankers of infected leaves (labeled X), and immediately extracted in 10 mL methanol, with overnight shaking (100 rpm) at room temperature. The methanol extracts were removed and saved, and the leaf segments were similarly re-extracted in an additional 10 mL methanol. The methanol extracts per sample were combined and evaporated at 40 °C with a Savant Speedvac Concentrator SVC 200H (Farmingdale, NY) to dryness. Extracts were dissolved in 0.5 mL dimethylsulfoxide. This extraction technique was also used for analyses of healthy leaves and canker diseased leaves of Ruby grapefruit from an established (≈10 years) commercial grove in Lake Wales, FL. Ten healthy and 10 cankered leaf samples were taken from each of 20 trees.

HPLC analyses. Compound profiles in extracts of healthy (H) and abraded (C) grapefruit leaves, and of leaf cankers (X) were analyzed by HPLC-MS and by HPLC-fluorescence detection, using a Varian HPLC with ProStar 210 pumps and a ProStar 410 autosampler controlled by Star software (ver. 6.41). Compound separations were achieved with a Waters XBridge C8 column (4.6 × 150 mm i.d.). Elution conditions included gradients of aqueous 0.5% formic acid/acetonitrile, initially composed of (90/10, v/v), and changed in composition in linear gradients to 80/20, 75/25, 60/40, 30/70, 30/70, 90/10, and 90/10 (v/v) at 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 Shimadzu RF 535 fluorescence HPLC detector. Fluorescence peak detection occurred at emission wavelengths of either 400 or 480 nm, with excitation wavelengths of either 330 or 388 nm, respectively. Fluorescence data were recorded and processed with PowerChrom 280 ver. 2.5 (eDAQ, Inc., Denistone East, Australia).

Results and Discussion

Detection of fluorescence peaks in the HPLC chromatograms of the grapefruit leaf extracts was optimal at two emission/excitation wavelength combinations. The chromatograms in Figure 2 show the fluorescence chromatograms of a methanol extract

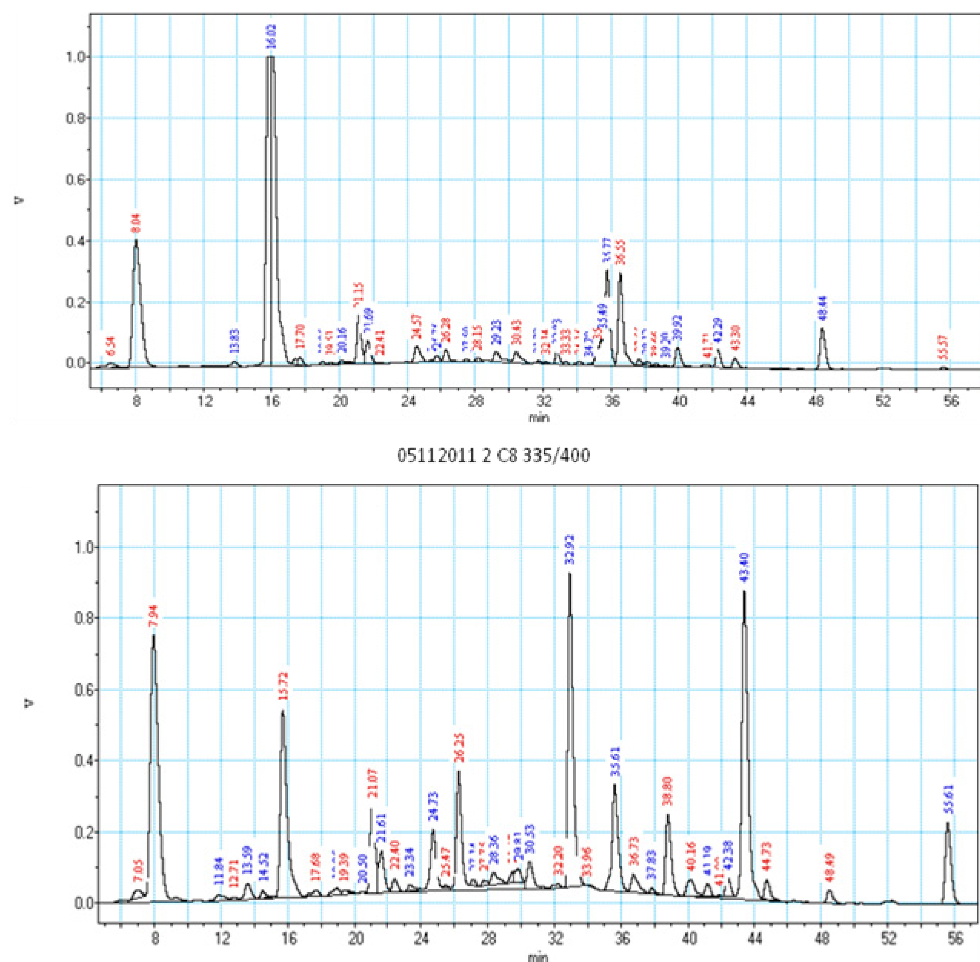

Fig. 2. Fluorescence chromatograms of a methanol extract of grapefruit leaf cankers obtained at both 338/480 nm (**top**) and at 335/400 nm (**bottom**).

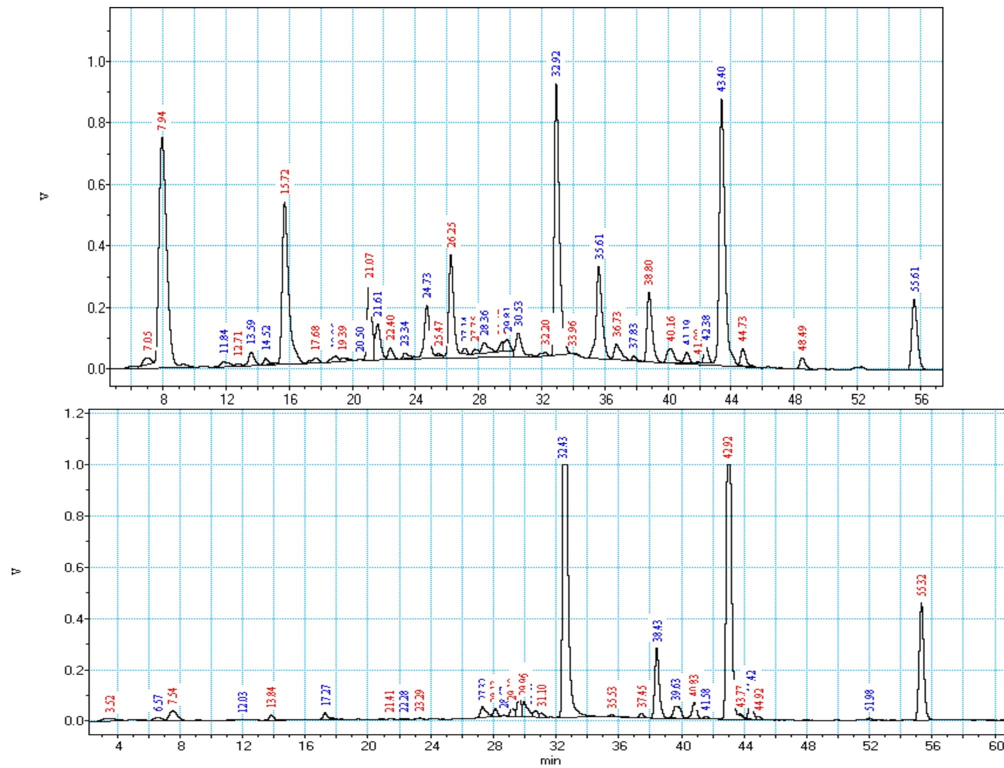

Fig. 3. Profiles of fluorescent compounds in the extracts of canker-infected leaves (**top**) and healthy, commercial grove leaves (**bottom**) at the 335/400 nm excitation/emission combination.

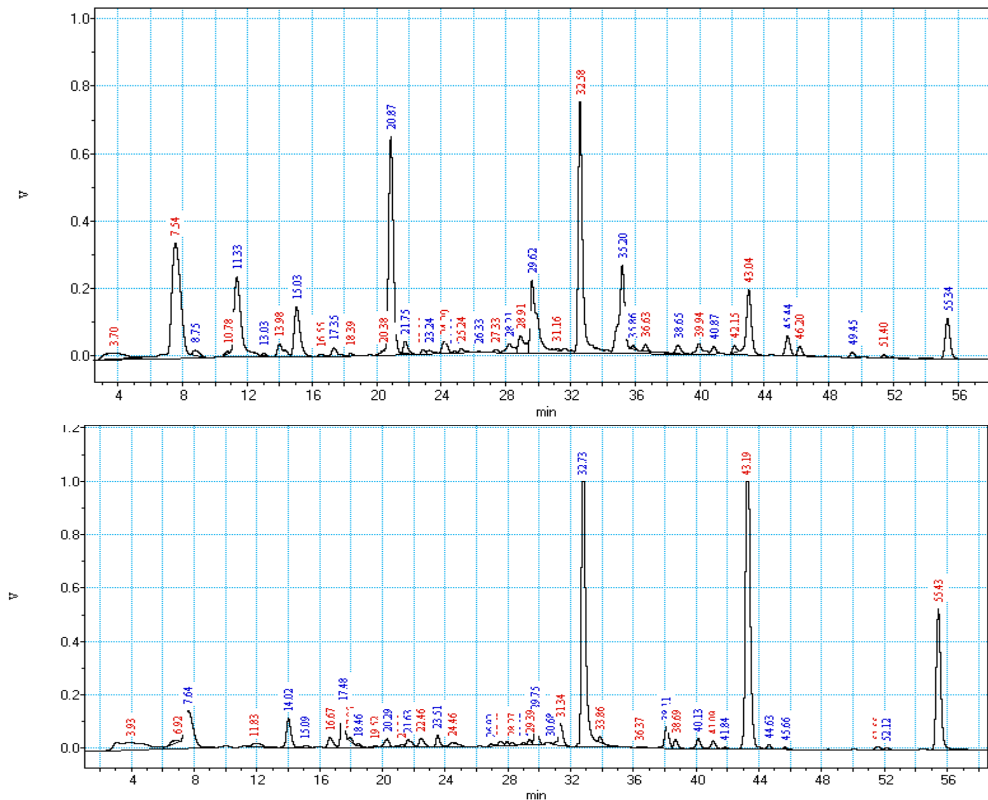

Fig. 4. Profiles of fluorescent compounds in extracts of canker-infected greenhouse leaves (**top**) and healthy grapefruit leaves (**bottom**).

of grapefruit leaf cankers measured at 338/480 nm (top) and at 335/400 nm (bottom). The fluorescence peaks eluting between 7 and 22 min were readily detected at both emission/excitation wavelength combinations, whereas major differences appeared in the detection of many of the later eluting peaks, particularly at 26.2, 32.9, 35.7, 43.4 and 55.6 min. These latter peaks were better detected at 335/400, although other minor peaks in this later-eluting portion of the chromatogram were better detected at 338/480. Hence, complete analyses of the differences in the fluorescence peak profiles between the H, C, and X leaf extracts need to be performed at both emission/excitation wavelength combinations.

The chromatograms in Figure 3 compare the profiles of fluorescent compounds in representative extracts of canker-infected (top) and healthy (bottom) commercial grove leaves at the 335/400 nm excitation/emission combination. Several important features include the fact that the two, prominent, early eluting peaks at 7.9 and 15.7 min in canker-infected leaf extracts are nearly absent in healthy leaf extracts. Other peaks between 21 and 26 min, and at 35.6 min are also prominent in the canker extract, but occur at much lower amounts in the healthy extracts. The three main later eluting peaks at 32.9, 43.4, and 55.6 min are in both samples, but occur at higher levels in the healthy leaf extracts.

Similar differences in fluorescent peak profiles also occurred in most of the greenhouse canker-infected and healthy leaves. Examples of a typical extract of canker-infected greenhouse leaves are shown in Figure 4 (top), and are compared to typical extracts of healthy grapefruit leaves (bottom). There is generally good agreement between observations made with commercial grove leaves and with greenhouse leaves; thus the latter are useful models for the study of changes in the chemical ecology attributable to infection by *Xcc*.

Initial quantitative comparisons were made of the prominent fluorescent peaks observed in the canker, control, and healthy leaves of the greenhouse-grown young grapefruit trees. Twenty-eight peaks were selected (labeled A to Z3) (Table 1) and were monitored as [Fluorescence Peak Area$_{335/400}$ per FW (g) extracted leaf tissue]. Control (C) (abraded, but not infected) leaf measurements included three collection dates and are averages of duplicate samples of 20 segments. Canker (X) (abraded and inoculated) leaf measurements included three collection dates with replicates of n = 3, 3, and 2 for collection dates 29 June 2010, 30 June 2010, and 6 July 2010, respectively. Healthy (H) leaf measurements included values of single samples obtained at 6 July 2010, and triplicate samples obtained on 30 June 2010. The 12 peaks in Table 1 highlighted in yellow are those that exhibit large increases in canker-infected leaves, and thus preliminarily represent compounds produced in response to the bacterial infection. These results provide an indication of the complexity of the chemical response in grapefruit to attack by *Xcc*.

It is also of interest to note that three other peaks, highlighted in turquoise, exhibit large decreases in the wounded, non-infected leaves (C) and the canker-infected (X) leaves. The physiological significance of these decreases in response to the tissue damage is unclear. Many phenolic flavonoids in grapefruit leaves were unresponsive to the abrasion and/or infection (data not shown), thus indicating a specificity for the compounds highlighted in Table 1 in the grapefruit responses to injury and bacterial infection.

Data collected from studies on chemical changes in citrus leaves infected with *Xcc* could lead to an understanding of how these changes affect the survival of the bacterium. It may be found that enhanced production of one or more of these compounds may help control the spread of the disease.

Table 1. Values of Fluorescence Peak Area$_{335/400}$ per FW (g) in healthy non abraded and non-infected leaves (labeled H), then from injured, but not infected leaves (labeled C), and then from cankers of infected leaves (labeled X). Peaks highlighted in yellow are those exhibiting large increases following Xcc infection. Peaks highlighted in turquoise are those exhibiting large decreases following physical abrasion from H to C modification.

Compound	Elution time (min)	H	C	X
A	7.8	614±125	1730±1152	6128±3645
B	10.2	90±18	328±158	132±80
C	11.3	45±12	142±71	100±32
D	11.7	57±22	121±60	1227±865
E	12.8	51±32	34±18	111±77
F	13.3	15±3	31±21	80±50
G	14.3	41±5	55±35	125±76
H	15.4	55±21	349±367	1418±829
I	15.7	928±1055	356±283	1906±1622
J	16.4	33±8	46±28	80±51
K	17.0	1114±618	638±618	809±812
L	18.2	73±11	70±38	51±33
M	18.8	10±9	34±21	124±93
N	21.5	149±58	208±105	2316±2293
O	22.3	18±15	40±43	280±171
P	22.9	137±38	224±122	222±120
Q	27.9	255±151	213±206	242±180
R	32.4	168±45	60±39	139±62
S	34.7	19±37	7±6	200±100
T	35.1	1±1	9±6	524±330
U	35.6	12±1	23±27	101±80
V	36.2	76±14	44±36	131±76
W	37.2	6±4	7±3	26±26
X	38.1	296±158	67±110	71±50
Y	42.0	0±0	9±14	122±57
Z	43.0	279±104	109±66	115±82
Z1	46.2	37±14	16±10	31±19
Z2	48.3	6±4	6±6	49±39
Z3	55.6	60±29	23±15	28±11

Literature Cited

Afex, U., J. Orenstein, S. Carmeli, V. Rodov, and M. Bar Joseph. 1999. Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*. Phytochemistry 50:1129–1132.

Afek, U., A. Sztejnberg, and S. Carmely. 1986. 6,7-Dimethoxycoumarin, a citrus phytoalexin conferring resistance against *Phytophthora gummosis*. Phytochemistry 8:1855–1856.

Baudoin, A.B.A.M. and J.W. Eckert, 1985. Development of resistance against *Geotrichum candidum* in lemon peel injuries. Phytopathology 75:174–179.

Brown, E.G. and C.R. Barmore. 1983. Resistance of healed citrus exocarp to penetration by *Penicilium digitatum*. Phytopathology 73:691–694.

D'hallewin, G., M. Schirra, M. Pala, and S. Ben-Yehoshua. 2000. Ultraviolet C irradiation at 0.4 kJ·m^{-2} reduced decay without causing damage or affecting postharvest quality of Star Ruby grapefruit (*C. paradisi* Macf.). J. Agr. Food Chem. 48:4571–4575.

Gotwald, T.R., J.H. Graham, and T.S. Schubert. 2002. Citrus canker: The pathogen and its impact. Online. Plant Health Progr. Doi:10.1094/PHP-2002-0812-01-RV.

Koizumi, M. 1983. Relationship between wound-healing process of citrus leaf tissues and their successful infection through wounds by *Xanthomonas campestris* pv. Citri (Hasse) Dye. Annu. Phytopathol. Soc. Japan 49:352–360.

Lai, S., A. Lai, R.R. Stange, Jr., T.G. McCollum, and M. Schirra. 2003. Characterization of the wound-induced material in *Citrus paradisi* fruit peel by carbon-13 CP-MAS solid state NMR spectroscopy. Phytochemistry, 63:177–183.

Ortuño, A., L. Díaz, N. Alvarez, I. Porras, A. García-Lidon, and J.A. Del Río. 2011. Comparative study of flavonoid and scoparone accumulation in different *Citrus* species and their susceptibility to *Penicilliuim digitatum*. Food Chem. 125:232–239.

Stange, Jr., R.R., S.L. Midland, J.W. Eckert, and J.J. Sims. 1993. An antifungal compound produced by grapefruit and Valencia orange after wounding of the peel. J. Nat. Prod., 56:1627–1629.