Incidence of Asiatic Citrus Canker on Trifoliolate Orange and Its Hybrid Accessions in a Florida Field Planting

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Additional index words. *Xanthomonas citri* pv. *citri*, resistance, Aurantioideae, Rutaceae

Abstract. Asiatic citrus canker (ACC) foliar lesions were evaluated on progenies of 84 seed-source genotypes (“parent genotypes”) from the Citrus Variety Collection (CVC) of the University of California at Riverside (UCR) of *Citrus trifoliata* and hybrids between *C. trifoliata* and other *Citrus* species and hybrids. Trees were planted Aug. 2013 in a completely randomized design at the Fort Pierce U.S. Department of Agriculture (USDA) grove. Plants were assessed visually Aug. 2017, Sept. 2019, and Sept. 2020 for distinctive ACC lesion incidence and severity. Progeny were compared by parent genotypes using nonparametric analysis. Incidence of ACC [percentage of leaves displaying symptoms, verified by quantitative polymerase chain reaction (qPCR) to be associated with *Xanthomonas citri* pv. *citri*] across parent genotypes ranged from 8% to 80% (mean, 49%) of leaves affected in 2017, from 4% to 58% (mean, 29%) in 2019, and 8% to 46% (mean, 25%) in 2020. In 2017, of 49 *C. trifoliata* parent genotypes, only four separated from the two highest ACC-incidence statistical categories [Citrus Research Center (CRC) 3345, 3484, 3888, and 4017], whereas 29 of the 35 *C. trifoliata* hybrids displayed lower ACC incidence, which separated from the two highest statistical categories. In 2019, of the *C. trifoliata*, only six separated from the highest ACC-incidence statistical category (CRC 3330, 3484, 3547, 3549, 3876, and 3888), whereas all 35 *C. trifoliata* hybrids displayed lower ACC incidence and separated from the highest statistical category, and 26 hybrids separated from 18 of the *C. trifoliata*. In 2020, only three *C. trifoliata* separated from the highest ACC-incidence statistical category (CRC 2861, 3549, and 3888) and 20 hybrids separated from 18 of the *C. trifoliata*. By parent genotype, ACC incidence correlated substantially between each pair of the 3 years, with r^2 values of 0.39, 0.57, and 0.65. Of 34 hybrids validated, similar numbers had *C. trifoliata*, grapefruit (*C. ×aurantium* var. *racemosa*), and sweet orange (*C. ×aurantium* var. *sinensis*) chloroplasts. Chloroplast type affected ACC incidence and severity, but not in a consistent manner. Near-isogenic groups within *C. trifoliata*, as determined by DNA markers, were associated with some statistically different ACC sensitivity. Overall, hybrids of *C. trifoliata* with other citrus types displayed markedly reduced ACC sensitivity compared with *C. trifoliata*, indicating that this trait is readily overcome through breeding.

Asiatic citrus canker (ACC, caused by *Xanthomonas citri* pv. *citri*, Xcc) is now widespread in Florida citrus (Gottwald et al., 2002; Irey et al., 2006). In the field, ACC is spread primarily by wind-driven rain, but can also be transferred by equipment and personnel, and affects foliage and fruit (Gottwald et al., 2002).

Highly susceptible plants often develop severe symptoms on leaves, twigs, and fruit, resulting in premature fruit and leaf drop, and marked scarring on harvested fruit. The use of

clean nursery stock produced in protective structures is typically the foundation of ACC control. At some locations where ACC is severe and fruit are grown for fresh use, windbreaks may be established to reduce spread by wind-driven rain. Once planted, trees receive frequent sprays with protective chemicals, and equipment and personnel entering and leaving groves may be subject to disinfection [reviewed by Gottwald et al. (2002)]. Economic losses from this disease may be sizable, and the use of

resistant cultivars is desirable to limit damage from ACC and to reduce costs for control.

This study was established to assess the relative huanglongbing (HLB) sensitivity of *Citrus trifoliata* (trifoliolate orange, previously *Poncirus trifoliata*) accessions and a diverse assortment of its hybrids, as some members of this species have been shown to be HLB tolerant in an earlier study (Ramadugu et al., 2016), and to confer HLB tolerance to many of its hybrids (e.g., Stover et al., 2015). Although showing low sensitivity to HLB, some trifoliolate orange hybrids have been reported to be very sensitive to ACC (e.g., Stover et al., 2013), potentially a negative trait transferred when breeding for HLB tolerance. Therefore, in our study, we assessed the severity of foliar ACC lesions on progeny from 84 parent genotypes of *C. trifoliata* and its hybrids exposed to naturally occurring Xcc inoculum in the field in East-Central Florida to identify their relative susceptibility to ACC.

Materials and Methods

Source, identity, and naming of plant material. Seeds for all progenies were provided by the USDA–Agricultural Research Service (ARS) National Clonal Germplasm Repository for Citrus and Dates at UCR, and were harvested from fruit of field-grown trees in the CVC of UCR (see <http://www.citrusvariety.ucr.edu>). Accessions in the CVC are designated with CRC numbers. The seedling progenies of 84 seed-source accessions were used in this study and are referred to by the CRC number of the seed parent accession. The online version of Table 1 provides access through hyperlinks to the history of each accession with taxonomic references, from the CVC (CRC) and the Germplasm Resource Information Network–Global of the USDA–ARS National Plant Germplasm System. Within the genus *Citrus*, incidence of nucellar embryony varies greatly [reviewed in Frost and Soost (1968)]; therefore, some of the plants we tested were essentially identical genetically to the female parent (seeds serving as clonal propagules), whereas others were half-sib hybrids of known seed parents but with undetermined pollen parents. As described later, the vast majority of these trees are expected to result from nucellar embryony. For convenience, they are referred to as “accessions,” using the CRC numbers of the seed source accessions. Hereafter, the seed source accessions providing the progeny used in this study are referred to as “parent genotypes.”

Plant development and maintenance. Seedling development, field planting, and general care were conducted as described in Richardson et al. (2011) and Westbrook et al. (2011), and were typical for citrus production, except with minimal pesticide application. Before field planting, seedlings were maintained in a greenhouse free of asian citrus psyllid [vector of *Candidatus Liberibacter asiaticus* (CLas), causal agent of HGB] and other arthropod pests. Plants were sown for germination in Jan. 2012, with some resown in June 2012 where germination was initially low. As a component of the

HLB-related investigation, half of the seedlings from each parent genotype were bud-inoculated with CLAs in the greenhouse in Oct. 2012, and all were field-planted during Aug. 2013. In our study, plants were evaluated for ACC 5 to 8 years after germination.

Trees were typically sprayed every 2 weeks with 435 horticultural oil and 200 ppm Magna-Bon (Magna-Bon II LLC, Okeechobee, FL). In a few instances, spraying was suspended for 2 months or more for insect observations in other studies. The nutritional component varied throughout the year and included a wide range of products. The list of materials included 20N-4.4P-16.6K, 14N-0.9P-11.6K, Keyplex 1400 DP (Keyplex, Winter Park, FL), Ocean Organics seaweed extract (Ocean Organics, Ann Arbor, MI), and N-Sure 28N-0P-0K (Tessenderlo Kerley, Inc., Phoenix, AZ). Other nutrients were included periodically, but none that were considered to have any effects on canker.

Assessing ACC sensitivity. Formation of foliar lesions indicative of ACC is affected by stage of leaf development when exposed to Xcc (Gottwald and Graham, 1992; Graham and Gottwald, 1991; Vernière et al., 2003). Wind-driven rain is the main factor in field spreading of inoculum (Graham et al., 1992), and the combination of conducive environmental conditions and available inoculum must coincide with foliage susceptibility for ACC development. For these reasons, we assessed ACC presence as both incidence (percentage of leaves displaying lesions) and severity (the percentage of leaf area affected by lesions). ACC symptoms were assessed in Aug. 2017, Sept. 2019, and Sept. 2020. The visual estimates of leaf area disease (ACC severity) were guided by a standard area diagram set prepared using image analysis as described in another study (Bock et al., 2010). In 2017, the overall percentage of leaf area displaying ACC lesions on each tree was assessed but was found to display little mean

separation; so, in 2019 and 2020, the percentage of leaf area displaying ACC lesions only on the most severely affected leaf was assessed on each tree.

Statistical analyses. We tested whether the incidence and severity of ACC differed among progenies of the 86 parent genotypes using Kruskal Wallis nonparametric analysis (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC). Regressions were conducted using Microsoft Excel (Microsoft Office version 2016; Microsoft Corporation, Redmond, WA).

qPCR verification of pathogen associated with symptoms. Verification that purported ACC lesions were in fact a result of infection with Xcc was conducted by qPCR analysis. Four trees were selected randomly for sampling of well-known accessions from each of pure *C. trifoliata*, citranges, citrumelos, US-119 (the only unifoliate hybrid in the trial), and four unifoliate from adjoining rows. Four leaves showing clear canker lesion symptoms were sampled from different branches on each tree. Lesions and the surrounding area, ≈ 200 mg of tissue, were excised and macerated for DNA extraction. Carrizo and Hamlin leaves from clean greenhouse trees, extracted in the same manner, were used as negative controls.

DNA was isolated using the ZR-Duet Quick-DNA/RNA Miniprep Plus Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. DNA concentration and purity were analyzed using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). qPCR was performed using the PowerUp SYBR Green master mix (Thermo Fisher Scientific) and 100 ng of template on the Applied Biosystems AB7500 Real-Time qPCR system (Thermo Fisher Scientific) according to the manufacturer's instructions. Primer sets (Cubero and Graham, 2002) were specific to the 16S ribosomal DNA sequence of *X. euvesicatoria* pv. *citrumelonis* [abbreviated hereafter as Xec; recent synonyms *X. alfalfae* pv. *citrumelonis* (Constantin et al., 2016), *X. axonopodis* ssp. *citrumelonis*, and *X. campestris* pv. *citrumelo* (National Center for Biotechnology Information, 2020)], *lrp* gene conserved in *Xanthomonas* spp., *pth* gene conserved in Xcc strains, and citrus *Glyceraldehyde 3-phosphate dehydrogenase* as a reference gene. DNA was isolated from liquid cultures of Xcc (strain ID 3213 from D. Gabriel) and Xec (strain ID 3048 from D. Gabriel) using phenol, phenol-chloroform-isoamyl alcohol (25:24:1), followed by two 70% ethanol washes. The dried pellet was then resuspended in 100 μ L 10 mM Tris buffer (pH 8.5) for use as positive controls. In addition to the clean citrus DNA controls described, standard water controls were also included.

Single nucleotide polymorphism analysis of chloroplasts. To determine chloroplast identities, comparisons were made using data on 462 well-distributed chloroplast single nucleotide polymorphisms (SNPs) included on a 50,000 + SNP chip developed at UCR with polymorphic SNPs selected based on sequences of 41 diverse accessions from the UCR CVC and public databases (Hiraoka, 2020).

Results and Discussion

ACC pathogen widespread in trial. ACC was ubiquitous in the research farm where this experiment was conducted. The lesions caused by Xcc infection initially have a distinct yellow halo, which often becomes necrotic, and the lesions feel sandpapery on the abaxial and adaxial surfaces of the leaves, and raised lesions form on the stems as well (Das, 2003). These traits were displayed in all trees throughout the planting. Virtually all lesions had progressed beyond the yellow halo stage in the trial planting, even though neighboring unifoliate citrus—citrus with a leaf composed of a single leaflet rather than the compound trifoliate (3-leaflet) leaf of *C. trifoliata* and its F1 hybrids—maintained many halos. The bacterium Xec, causal agent of *Citrus bacterial spot*, is also present in Florida and produces similar symptoms, but is generally reportedly prevalent only in nurseries and is associated with frequent mechanical injury (budding and pruning) to trees [reviewed in Graham and Gottwald (1991)]. More recently, there are unpublished reports that the HLB-tolerant cultivar LB8-9 (Sugar-Belle®) is highly susceptible to Xec but not Xcc, but lesions are only found on leaves and never fruit (F.G. Gmitter, Jr., personal communication). For our study, qPCR with primers to distinguish the two *Xanthomonas* species (data not shown) was run on ACC-symptomatic leaves from the test plot and adjoining rows, and all tested strongly positive for Xcc [all but two with a cycle threshold (Ct) <25, one at Ct 30.9 and one at Ct 26.0 (mean, 22.6), reflecting a mean copy number of 240,000 per ng plant DNA]; only 44% had detectable Xec and then at very low titers (Ct, >35.4; a mean copy number of 4 per ng DNA). Therefore, the distinctive canker lesions were attributed to Xcc.

ACC sensitivity in accessions. CLAs inoculation before planting had no effect on ACC, thus all plants of an accession were assessed as a single group (data not shown). Almost all accessions with a lower ACC lesion incidence were *C. trifoliata* hybrids rather than pure *C. trifoliata* in all 3 years of data collection (Table 1). To aid in visualizing the responses across all accessions, pure *C. trifoliata* accessions are in bold in the leftmost column of Table 1; in each year for incidence and severity of ACC, the accession means that separated statistically are shaded in gray for low incidence and in bold for high incidence.

Incidence of ACC across parent genotypes ranged from 8% to 80% (mean, 49%) of leaves affected in 2017, from 4% to 58% (mean, 29%) in 2019, and 8% to 46% (mean, 25%) in 2020. In 2017, of 49 *C. trifoliata* parent genotypes, only four separated from the two highest ACC-incidence statistical categories (CRC 3345, 3484, 3888, and 4017), whereas 29 of the 35 *C. trifoliata* hybrids displayed lower ACC incidence, which separated from the two highest statistical categories (the two highest were used for this comparison because so few accessions were in the highest group). In 2019, of the *C. trifoliata*,

Received for publication 4 Jan. 2021. Accepted for publication 9 Feb. 2021.

Published online 3 May 2021.

We thank Ellen Cochrane for greenhouse care; Sean Reif, Jefferson Shaw, and Diego Garcia for data collection in the field; Stephen Mayo for overseeing tree general maintenance; and Spencer Marshall for quantitative polymerase chain reaction (qPCR) diagnostics. Dr. Dean Gabriel kindly provided a culture of *X. euvesicatoria* pv. *citrumelonis* for use as a qPCR positive control. This research was originally presented at the 2020 annual meeting of the Florida State Horticultural Society.

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Table 1. Incidence and severity of asian citrus canker (ACC) in leaves of half progenies of 84 seed source accessions exposed to field infection in Ft. Pierce, FL, for 3 years of assessment. In each column, to assist visualization, means with solid gray fill are in the least ACC-sensitive statistical category and are significantly different from those in bold.

Name of seed parent ^a	CRC no. ^b	NPGS no. ^c	2017				2019				2020			
			Seed parent (reported)	Pollen parent (reported)	Seed parent (by chloroplast genome) ^w	Trifoliolate category ^y	Incidence of ACC lesions (% of all leaves)	Severity of ACC lesions (% of all leaves)	Incidence of ACC lesions (% of all leaves)	Severity of ACC lesions (% of all leaves)	Incidence of ACC lesions (% of all leaves)	Severity of ACC lesions (% of all leaves)	Incidence of ACC lesions (% of all leaves)	Severity of ACC lesions (% of all leaves)
Citrandarin	2618	PI 539839	Trifoliolate	Mandarin	Trifoliolate	Citrandarin	8.0 f ^z	10.0 ab	15.0 b-g	10.0 cd	26.0 a-e	11.0 a-c	8.0 e	9.0 bc
<i>Poncirus trifoliata</i> monoembryonic														
<i>Poncirus trifoliata</i> × <i>Satsuma</i>	3888	PI 433263	Trifoliolate	na	nd	Citrandarin	10.0 ef	5.0 ab	15.0 b-g	10.0 cd	8.0 e	11.1 a-c	17.6 c-d	11.1 a-c
Unnamed (<i>P. trifoliata</i> × <i>Satsuma</i>)	3881	PI 433930	Trifoliolate	Satsuma	nd	Citrandarin	15.0 ef	6.9 ab	11.8e-g	12.9 cd	17.6 c-d	17.0 a-c	8.0 e	11.1 a-c
Swingle (CPB 4475 Citrumelo)	3341	PI 539824	Grapefruit	Trifoliolate	Grapefruit	Citrumelo	20.0 d-f	10.0 ab	4.0 g	6.0 d	12.0 e	15.1 a-c	12.0 e	15.1 a-c
Swingle	3771	PI 539828	Grapefruit	Trifoliolate	Grapefruit	Citrumelo	20.0 ef	7.5 ab	10.7 fg	9.8 d	12.0 e	15.1 a-c	15.7 de	10.5 a-c
X639	3957	PI 300256	Mandarin	Trifoliolate	Trifoliolate	Citrandarin	21.0 d-f	5.5 ab	6.2 g	7.9 d	15.3 de	10.5 a-c	12.4 e	9.3 bc
Citangor	1447	PI 539836	Trifoliolate × orange	Orange	Trifoliolate	Citrange	21.4 d-f	6.4 ab	11.0 fg	10.7 cd	12.4 e	10.2 a-c	16.1 de	13.9 a-c
Swingle	3767	PI 539844	Grapefruit	Trifoliolate	Grapefruit	Citrumelo	21.4 d-f	7.1 ab	11.3 fg	9.9 cd	16.3 de	10.2 a-c	14.6 e	10.0 a-c
Yuma	3205	PI 539816	Trifoliolate	Grapefruit	Grapefruit	Complex	24.5 d-f	9.1 ab	10.3 fg	12.0 cd	16.1 de	11.6 a-c	34.5 ab	7.3 c
US 119	3998	PI 539832	Grapefruit × trifoliolate	Orange	Grapefruit	Citrange	27.2 d-f	7.2 ab	10.8 fg	10.6 cd	34.5 ab	18.0 cd	28.6 b-e	8.0 bc
C-35	3912	PI 539821	Orange	Trifoliolate	Orange	Trifoliolate	28.8 d-f	6.3 ab	38.0 ab	18.0 cd	28.6 b-e	11.1 e	12.0 e	13.1 a-c
Small Leaf														
Frost tetraploid														
Carrizo	3484	PI 539775	Trifoliolate	na	nd	Trifoliolate	29.5 d-f	6.0 ab	10.7 fg	9.7 d	11.1 e	10.9 a-c	19.7 c-e	11.2 a-c
Sacaton	2863	PI 150916	Orange	Trifoliolate	Orange	Citrange	29.6 d-f	6.3 ab	15.0 c-g	15.8 b-d	12.0 e	10.9 a-c	16.8 de	12.7 a-c
Rusk	3337	PI 539823	Trifoliolate	Grapefruit	Grapefruit	Citrumelo	31.3 d-f	7.5 ab	9.3 fg	10.1 d	12.3 c	10.8 a-c	18.3 c-e	14.3 a-c
Benton	1441	PI 539809	Orange	Trifoliolate	Orange	Citrange	31.4 d-f	7.9 ab	9.7 fg	9.1 d	13.2 e	10.3 a-c	14.7 e	11.0 a-c
Christiansen														
Morton	3345	PI 539771	Trifoliolate	na	nd	Trifoliolate	31.7 d-f	6.7 ab	43.7 ab	22.3 a-c	59.5 a	19.5 ab	17.3 c-e	10.2 bc
Hall grapefruit × <i>P. trifoliata</i>	2748	PI 539754	Trifoliolate	Orange	Trifoliolate	Citrange	32.7 d-f	5.5 ab	14.8 d-g	12.3 c	16.8 de	12.7 a-c	14.4 e	9.8 a-c
C-32	3821	PI 539845	Grapefruit	Trifoliolate	Grapefruit	Citrumelo	32.8 d-f	7.2 ab	26.4 b-e	16.8 cd	29.5 a-e	10.8 a-c	15.8 de	10.3 a-c
Troyer	1459	PI 539820	Orange	Trifoliolate	Orange	Citrange	33.8 d-f	5.8 ab	8.8 g	8.1 d	18.3 c-e	10.8 a-c	14.3 a-c	10.3 a-c
Sanford	276	PI 539814	Trifoliolate	Orange	Orange	Citrange	34.2 d-f	7.1 ab	8.8 g	10.5 cd	15.8 de	11.3 a-c	14.0 e	11.0 a-c
African shaddock × Rubidoux trifoliolate	3969	PI 539830	Pummelo	Trifoliolate	Pummelo	Citrumelo	35.4 d-f	6.7 ab	9.0 g	9.9 cd	14.7 e	10.2 bc	14.4 e	9.8 a-c
Morton	1463	PI 539811	Trifoliolate	Orange	Trifoliolate	Citrange	35.9 d-f	7.3 ab	8.2 g	8.3 d	17.3 c-e	10.8 a-c	14.4 e	9.8 a-c
Rusk	301	PI 13002	Orange	Trifoliolate	Trifoliolate	Citrange	36.1 d-f	6.7 ab	10.8 fg	9.7 d	17.3 c-e	10.8 a-c	14.4 e	9.8 a-c
Trifeola	3954	PI 539846	Grapefruit × mandarin	Trifoliolate	Grapefruit	Citangor	36.1 d-f	9.4 ab	21.7 b-f	15.2 cd	14.4 e	10.0 a-c	19.8 c-e	11.3 a-c
Spanish sweet × <i>P. trifoliata</i>	3336	PI 539817	Orange	Trifoliolate	Orange	Citrange	37.2 d-f	6.7 ab	13.7 d-g	12.8 cd	21.9 b-e	10.8 a-c	16.5 a-c	15.6 a-c
Uvalde	2865	PI 150917	Trifoliolate	Trifoliolate	Trifoliolate	Citrange	40.5 d-f	7.5 ab	17.6 b-g	16.1 cd	21.9 c-e	12.5 a-c	11.0 e	7.0 c
C-190	3889	PI 539829	Grapefruit	Trifoliolate	Grapefruit	Citrumelo	40.6 d-f	10.0 ab	12.8 e-g	8.8 d	16.8 c-e	11.3 a-c	16.8 c-e	15.6 a-c
Citradia	1436	PI 539833	Trifoliolate	Sour	Trifoliolate	Citradia	40.9 d-f	7.7 ab	16.8 b-f	11.6 cd	16.5 de	16.5 a-c	21.4 b-e	15.6 a-c
Citrange #1416	2866	PI 539815	Trifoliolate	Orange	Orange	Citrange	42.9 c-f	9.3 ab	6.6 g	9.0 d	21.4 b-e	12.5 a-c	33.0 a-c	14.0 a-c
Citron 1449	1449	PI 539838	Trifoliolate	Lemon	Lemon	Citron	45.7 c-f	12.0 ab	14.6 d-g	12.5 cd	21.9 c-e	10.4 a-c	26.6 a-e	10.4 a-c
Sacaton	3414	PI 539826	Trifoliolate	Grapefruit	Grapefruit	Citrumelo	46.0 b-f	7.0 ab	17.6 b-g	13.2 cd	11.0 e	12.3 a-c	29.3 a-d	12.3 a-c
Rich 7-5	3587	PI 539785	Trifoliolate	na	nd	Trifoliolate	46.3 b-f	5.0 b	44.5 ab	25.0 a-c	33.0 a-c	14.0 a-c	29.3 a-d	12.3 a-c
Argentina	3876	PI 539788	Trifoliolate	na	nd	Trifoliolate	46.4 b-f	7.1 ab	45.5 ab	15.8 cd	33.0 a-c	14.0 a-c	29.3 a-d	12.3 a-c
Kryder 60-2	3206	PI 539757	Trifoliolate	na	nd	Trifoliolate	46.7 b-f	11.7 ab	58.0 a	40.3 a	31.5 a-e	10.8 a-c	20.1 c-e	11.7 a-c
Simmons	3213	PI 539763	Trifoliolate	na	nd	Trifoliolate	48.3 b-f	8.6 ab	32.5 a-c	19.0 a-c	20.3 b-e	11.7 a-c	38.5 ab	17.5 a-c
Citrumelo 1452	3549	PI 539780	Trifoliolate	na	nd	Trifoliolate	48.6 b-f	8.6 ab	31.9 bc	20.1 bc	20.3 b-e	11.7 a-c	38.5 ab	17.5 a-c
Towne "F"	1452	PI 539822	Trifoliolate	Grapefruit	Grapefruit	Citrumelo	50.0 b-f	11.3 a	13.3 d-g	12.7 cd	20.3 b-e	11.7 a-c	38.5 ab	17.5 a-c
Savage	3572	PI 539783	Trifoliolate	na	nd	Trifoliolate	51.0 b-f	9.0 ab	51.6 a	34.8 a	18.3 c-e	15.5 a-c	35.2 a-d	16.8 a-c
Cunningham	275	PI 539813	Trifoliolate	Orange	Trifoliolate	Citrange	51.0 b-e	7.5 ab	13.7 d-g	10.0 cd	28.9 a-e	15.5 a-c	30.8 a-d	15.5 a-c
Jacobson	271	PI 539812	Trifoliolate	Orange	Orange	Citrange	53.2 b-e	10.0 ab	45.2 ab	30.4 ab	27.8 ab	12.8 a-c	39.0 a-c	41.8 ab
Kryder 28-3	3411	PI 539773	Trifoliolate	na	nd	Trifoliolate	53.3 b-e	12.5 ab	42.0 ab	28.4 ab	31.7 a-c	15.8 a-c	41.8 ab	12.0 a-c
Little-leaf	3219	PI 539767	Trifoliolate	na	nd	Trifoliolate	54.0 b-e	8.3 ab	37.1 ab	27.4 ab	31.7 a-c	15.8 a-c	41.8 ab	12.0 a-c
Kryder 15-3	4008	PI 600649	Trifoliolate	na	nd	Trifoliolate	56.3 b-e	12.5 a	45.3 a-c	31.7 a-c	39.0 a-c	15.8 a-c	41.8 ab	12.0 a-c
Hiryu	3217	PI 539765	Trifoliolate	na	nd	Trifoliolate	56.7 a-e	8.3 ab	51.7 a	33.2 ab	14.8 de	12.0 a-c	41.8 ab	12.0 a-c
Citrumelo	3795	PI 539787	Trifoliolate	Grapefruit	Grapefruit	Citrumelo	56.7 a-e	10.0 ab	18.3 b-f	14.3 cd	14.8 de	12.0 a-c	41.8 ab	12.0 a-c
	3348	PI 539825	Trifoliolate	Grapefruit	Grapefruit	Citrumelo	57.5 a-d	10.0 ab	18.3 b-f	14.3 cd	14.8 de	12.0 a-c	41.8 ab	12.0 a-c

(Continued on next page)

Table 1. (*Continued*) Incidence and severity of asian citrus canker (ACC) in leaves of half progenies of 84 seed source accessions exposed to field infection in Ft. Pierce, FL, for 3 years of assessment. In each column, to assist visualization, means with solid gray fill are in the least ACC-sensitive statistical category and are significantly different from those in bold.

Name of seed parent [†]	CRC no. [‡]	NPGS no. [§]	2017				2019				2020	
			Seed parent (reported)	Pollen parent (reported)	Seed parent (by chloroplast genome) [¶]	Trifoliolate category [‡]	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% all leaf area)	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% area of worst affected leaf)	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% area of worst affected leaf)
Kryder 8-5	3218	PI 539766	Trifoliolate	na	nd	Trifoliolate	58.0 a-d	8.5 ab	51.2 a	30.8 ab	36.3 ab	15.0 a-c
Pomeroy	1717	PI 539751	Trifoliolate	na	nd	Trifoliolate	58.1 a-d	12.5 a	46.4 a	34.8 a	27.3 a-e	11.5 a-c
S-302 Citranguma	3415	PI 539841	Trifoliolate × orange	Satsuma	Grapefruit	Citrandarin	58.3 a-d	8.9 ab	12.3 e-g	9.9 d	14.2 e	9.4 bc
Towne "G"	3207	PI 539758	Trifoliolate	na	nd	Trifoliolate	60.0 a-d	10.0 ab	44.5 ab	34.5 ab	37.0 ab	16.5 a-c
Benecke	3338	PI 539770	Trifoliolate	na	nd	Trifoliolate	60.0 a-d	12.0 a	51.0 a	29.0 ab	35.2 a-c	14.2 a-c
Kryder Medium	3212	PI 539762	Trifoliolate	na	nd	Trifoliolate	60.0 a-d	11.3 ab	50.0 a	34.8 a	29.0 a-e	13.2 a-c
Big-leaf	4006	PI 600647	Trifoliolate	na	nd	Trifoliolate	60.8 a-d	12.5 a	56.8 a	34.7 a	39.8 ab	18.0 a-c
Swingle	4138	RCRC4138	Trifoliolate	na	nd	Trifoliolate	61.3 a-d	11.3 ab	44.5 ab	25.0 a-c	39.0 ab	21.3 a
Australian	3151	PI 240121	Trifoliolate	na	nd	Trifoliolate	61.3 a-d	8.4 ab	44.9 ab	31.5 ab	24.6 a-e	14.3 a-c
USDA	1498	PI 539750	Trifoliolate	na	nd	Trifoliolate	61.4 a-d	10.9 ab	53.3 a	30.9 ab	30.1 a-e	13.1 a-c
Yamaguchi	3412	PI 539774	Trifoliolate	na	nd	Trifoliolate	62.2 a-d	10.6 ab	58.4 a	37.4 a	39.3 ab	15.1 a-c
Webber-Fawcett #22	2552	PI 539752	Trifoliolate	na	nd	Trifoliolate	62.3 a-d	9.5 ab	44.1 ab	29.0 ab	34.6 a-c	17.5 a-c
Rich 22-2	3211	PI 539761	Trifoliolate	na	nd	Trifoliolate	64.1 a-d	6.8 ab	36.8 ab	26.4 a-c	29.9 a-e	14.6 a-c
#27	3938	PI 539789	Trifoliolate	na	nd	Trifoliolate	65.0 a-c	8.1 ab	43.0 ab	27.1 ab	26.0 a-e	10.8 a-c
Little-leaf	4007	PI 600648	Trifoliolate	na	nd	Trifoliolate	65.6 a-c	8.8 ab	47.5 ab	29.0 ab	30.5 a-e	16.2 a-c
Texas	2861	PI 539755	Trifoliolate	na	nd	Trifoliolate	66.0 a-c	9.0 ab	51.0 a	34.2 ab	21.4 b-e	6.6 c
Kryder 5-5	3586	PI 539784	Trifoliolate	na	nd	Trifoliolate	66.7 a-c	13.9 a	41.3 ab	33.5 ab	31.0 a-e	12.2 a-c
Taylor	3571	PI 539782	Trifoliolate	na	nd	Trifoliolate	67.5 a-c	8.8 ab	39.4 ab	28.5 ab	33.4 ab	16.3 a-c
Fairhope	3351	PI 539772	Trifoliolate	na	nd	Trifoliolate	67.5 a-c	9.2 ab	44.5 ab	30.8 ab	25.3 a-e	12.0 a-c
Florida	2862	PI 539756	Trifoliolate	na	nd	Trifoliolate	67.5 a-c	8.3 ab	48.8 a	31.5 ab	30.2 a-e	9.7 a-c
Flying Dragon	3330	PI 539768	Trifoliolate	na	nd	Trifoliolate	67.7 a-c	9.5 ab	27.7 bc	19.6 a-c	29.2 a-e	11.4 a-c
Kryder 55-5	3215	PI 539764	Trifoliolate	na	nd	Trifoliolate	67.9 a-c	9.3 ab	43.4 ab	28.0 ab	28.3 a-e	12.0 a-c
#26 (Nanjing)	3939	PI 539790	Trifoliolate	na	nd	Trifoliolate	68.0 a-c	9.5 ab	49.0 a	34.5 ab	34.2 a-c	13.4 a-c
Rich 16-6	3485	PI 539776	Trifoliolate	na	nd	Trifoliolate	68.6 a-c	9.5 ab	37.9 ab	29.7 ab	34.9 a-d	15.9 a-c
Hiryu	3882	PI 433262	Trifoliolate	na	nd	Trifoliolate	70.0 a-c	8.3 ab	50.7 a	33.3 ab	30.3 a-e	17.3 a-c
Kryder 16-6	3210	PI 539760	Trifoliolate	na	nd	Trifoliolate	70.5 ab	8.2 ab	48.9 a	32.0 ab	32.1 a-e	14.5 a-c
Kryder 55-1	3486	PI 539777	Trifoliolate	na	nd	Trifoliolate	70.6 ab	8.8 ab	39.1 ab	46.0 a	16.3 a-c	16.3 a-c
P. trifoliata #22	4172	PI 658365	Trifoliolate	na	nd	Trifoliolate	70.7 ab	11.4 ab	54.2 a	29.7 ab	30.8 a-e	19.0 a-c
Barnes	2554	PI 539753	Trifoliolate	na	nd	Trifoliolate	71.0 ab	7.5 ab	42.8 ab	29.2 ab	30.0 a-e	11.7 a-c
Rich 12-2	3209	PI 539759	Trifoliolate	na	nd	Trifoliolate	74.1 ab	11.4 ab	45.2 a	31.6 ab	37.8 ab	15.5 a-c
English large	3548	PI 539779	Trifoliolate	na	nd	Trifoliolate	76.3 ab	10.6 a	48.3 ab	30.3 ab	31.3 a-e	12.9 a-c
Ronsee	3570	PI 539781	Trifoliolate	na	nd	Trifoliolate	76.4 ab	10.7 a	47.6 a	33.4 ab	34.1 a-c	13.7 a-c
Benoit	3547	PI 539778	Trifoliolate	na	nd	Trifoliolate	77.5 ab	7.5 ab	22.5 b-f	18.0 a-c	32.0 a-e	11.0 a-c
Marks	3588	PI 539786	Trifoliolate	na	nd	Trifoliolate	78.6 a	10.7 ab	55.3 a	34.9 a	45.4 a	23.7 a
Rubidoux	838	PI 539791	Trifoliolate	na	nd	Trifoliolate	80.0 a	10.0 ab	51.4 a	26.0 ab	33.0 a-d	12.5 a-c

[†]Name of seed source accession as indicated in the University of California (UC) Riverside Citrus Variety Collection (CVC). The old name *Poncirus trifoliata* is currently retained on the CVC website. It is now *C. trifoliata*.

[‡]Accession number as indicated in the UC Riverside CVC.

[§]PI number as indicated in the National Plant Germplasm System (NPGS).

^vType of chloroplast in seed source accessions (and thus seedlings), as determined by single nucleotide polymorphism analysis.

^wCitruselo is a grapefruit and *C. trifoliata* cross, citrange is a sweet orange and *C. trifoliata* cross, and citrandarin is a mandarin and *C. trifoliata* cross.

^xWithin a column, means followed by the same letter are not statistically different at $P < 0.05$.

USDA = U.S. Department of Agriculture; na = not applicable; nd = not determined.

only six separated from the highest ACC-incidence statistical category (CRC 3330, 3484, 3547, 3549, 3876, and 3888), whereas all 35 *C. trifoliata* hybrids displayed lower ACC incidence and separated from the highest statistical category, and 26 hybrids separated from 18 of the *C. trifoliata*. In 2020, only five *C. trifoliata* separated from the highest ACC-incidence statistical category (CRC 2861, 3484, 3549, 3888, and 4008) and 20 hybrids separated from 18 of the *C. trifoliata*. *C. trifoliata* accessions CRC 3484, 3549, and 3888 are noteworthy for their separation from the highest ACC-incidence category in 2 or 3 years. Only CRC 3888 had this distinction in all 3 years, but it should be noted that there was only one tree surviving and, because its parent accession is reported to be monoembryonic, it is likely a self-pollinated seedling or a hybrid. It was among the lowest accessions in canker sensitivity by all metrics in all 3 years and merits further investigation. We will propagate this one tree and test for ACC sensitivity in a replicated trial against appropriate standards. It is reported that in open-pollinated seedlings of *C. trifoliata* accessions at the UCR CVC, all zygotic seedlings were selfs or crosses between *C. trifoliata* accessions, likely a result of earlier flowering compared with other citrus (Kepiro and Roose, 2009). If this is a self or a hybrid with another trifoliolate, it could prove useful in understanding more fully the genetics of ACC sensitivity.

By accession means, ACC incidence correlated between each pair of the 3 years, with r^2 values of 0.39, 0.57, and 0.65 (Table 2). Within each year, metrics of ACC severity correlated with incidence of ACC, with r^2 values of 0.26, 0.94, and 0.42 for 2017, 2019, and 2020, respectively.

ACC sensitivity within citrumelos and citranges. Citrumelo, the name given to hybrids of grapefruit and trifoliolate orange, is widely used as citrus rootstock, and ‘Swingle’ citrumelo has been the most widely used citrus rootstock in Florida for the past 30 years (Florida Department of Agriculture and Consumer Services, 2016, 2020). Three of the 10 citrumelo accessions in our trial are independent entries of ‘Swingle’ (CRC 3341, 3771, and 3767), which were verified by SNP analysis, and all were consistently among the least ACC-sensitive accessions (Table 1). Most citrumelo accessions did not separate from the least ACC-sensitive category across all 3 years (9 in 2017, 8 in 2019, and all 10 in

2020). Two citrumelos were among the most ACC-sensitive accessions in this trial in some but not all years (CRC 3348 in 2017, and CRC 3821 in 2019 only), showing significant mean separations for ACC incidence. All citrumelos in the trials had a grapefruit seed parent.

Citrango, the name given to hybrids of sweet orange and trifoliolate orange, is also widely used as citrus rootstock, and ‘Carrizo’/‘Troyer’ (shown to be the same genotype) is the most widely used citrus rootstock in California and second-most widely used in Florida for the past 30 years (Florida Department of Agriculture and Consumer Services, 2016, 2020). Of the 14 citrange accessions, ‘Morton’ and ‘Rusk’ were duplicated, and ‘Carrizo’ matched ‘Troyer’ by SNP analysis (Roose unpublished). By chloroplast analysis, 10 accessions had a sweet orange seed parent and four trifoliolate orange. In no year and by no metric were any citranges statistically different from each other in ACC sensitivity (Table 1). Only in 2017 did any citrange fail to fall in the least ACC-sensitive statistical grouping (CRC 271 and 275).

ACC sensitivity by *C. trifoliata* subgroup. There are four near-isogenic groupings of *C. trifoliata* accessions, designated as restriction fragment length polymorphism (RFLP) types 1 through 4 by Fang et al. (1997), based on an array of molecular methods, and more recently verified by high-density SNP analysis (Roose, unpublished). Among the less ACC-sensitive *C. trifoliata* accessions, CRC 3888 (lowest sensitivity in all 3 years) and CRC 4017 are the only two accessions in this study from the uncommon RFLP type 2, whereas CRC 3484 and CRC 3549 are in the major group RFLP type 3, corresponding to the established “small flower trifoliolate” group. For the other common *C. trifoliata* designated as RFLP type 4 (corresponding to large flower trifoliate), only CRC 3345 displayed ACC incidence that separated from the two most severe statistical groupings, and only in 2017. CRC 3938 was the only RFLP type 1 accession, and it was in the highest ACC sensitivity group in all 3 years for all metrics. Statistical analysis of all *C. trifoliata* accessions by RFLP types showed type 2 to be significantly less sensitive than other types for ACC incidence only in 2017 (Table 3). A separate analysis comparing types 3 and 4 only (because they were much more numerous) showed type 3 accessions to be less

sensitive by metrics of ACC severity in all 3 years and by ACC incidence only in 2019.

ACC severity showed less robust mean separation in all 3 years (Table 1) and seemingly provided no noteworthy additional inferences. Because only a few accessions displayed statistically different means in 2017, these data are excluded from Table 1, but are presented here: Citrumelo CRC 1452 and trifoliate CRC 1717, 3338, 3548, 3570, 3586, 4006, and 4008 had a significantly greater percentage of canopy covered by lesions (10.6% to 13.9%) than trifoliolate CRC 3587 (5.0%).

ACC sensitivity as affected by chloroplast type. The UCR database on SNPs permitted assessment of the actual seed parent of the hybrids, as the cytoplasm is largely derived from the maternal parent in citrus (Abkenar et al., 2004). Eleven of the hybrids were the reciprocal cross from that expected, with the “female first” listing rule not observed. These are indicated by bold text in the “Seed parent (by chloroplast genome)” column (Table 1). ‘Yuma’ was previously found to be a citrumelo rather than a citrange (Xiang and Roose, 1988), and has a grapefruit chloroplast. Of the 34 hybrids with chloroplasts validated, similar numbers of field seedlings had trifoliolate, grapefruit, and sweet orange chloroplasts (74, 91, and 91, respectively), permitting the assessment of chloroplast influence on ACC sensitivity. The analyses were conducted in two ways: 1) by evaluating trifoliolate vs. grapefruit vs. sweet orange chloroplasts separately or 2) by combining sweet orange and grapefruit chloroplasts into a “pummelo” chloroplast category, because both sweet orange and grapefruit chloroplasts are known to be of pummelo origin (Nicolosi et al., 2000), although they are somewhat different from each other (Carbonell-Caballero et al., 2015). In all years, chloroplast type significantly but modestly affected ACC incidence and/or severity, but not in a consistent manner (Table 4). This analysis was conducted because Omar et al. (2017) reported that replacing the grapefruit chloroplast with that of ‘Meiwa’ kumquat reduced ACC sensitivity markedly. ‘Meiwa’ is markedly ACC insensitive, but sweet orange is only somewhat less ACC sensitive compared with grapefruit and trifoliolate orange.

ACC sensitivity comparison with previous studies. In an earlier study of more diverse citrus accessions, we reported incidence of ACC in Sept. 2010 and 2012 (Stover et al., 2013). That study included two shared *C. trifoliata* accessions (CRC 4007 and 3549) and three shared *C. trifoliata* hybrid accessions (CRC 301, 3771, 3957), but results were rather different. The only statistically significant difference among these accessions in 2010 and 2012 was that CRC 3771 (‘Swingle’ citrumelo) had a significantly greater reported ACC incidence (Sept. 2010, 62% of leaves vs. 34% to 37%; Sept. 2012, 28% vs. 6% to 11%) than the other accessions, and indeed was among the highest in the entire trial. In contrast, in 2017 and 2019, CRC 3771 was significantly lower in canker incidence compared with one or both

Table 2. r^2 Values from regressions of incidence and severity of asian citrus canker (ACC) in leaves of half progenies of 84 seed source accessions exposed to field infection in Ft. Pierce, FL, for 3 years (2017, 2019, and 2020) of assessment. Statistical significance of all regressions exceeds $P < 10^{-7}$.

Incidence of canker	SOC ^z , 2017	IOC ^y , 2019	SOC, 2019	IOC, 2020	SOC, 2020
2017	0.26	0.57		0.39	
2019			0.94	0.65	
2020					0.42

^zSOC is severity of canker lesions in the indicated year, measured as a percentage of the total canopy per tree covered by ACC lesions in 2017 and the percentage of the most symptomatic leaf per tree in 2019 and 2020.

^yIOC is incidence of canker lesions in the indicated year, measured as a percentage of all leaves on a tree showing ACC lesions.

Table 3. Association with restriction fragment length polymorphism (RFLP) type for incidence and severity of asian citrus canker (ACC) in leaves of progenies of 49 seed source *C. trifoliata* accessions exposed to field infection in Ft. Pierce, FL, for 3 years (2017, 2019, and 2020) of assessment.

RFLP type ^z	N, 2020	2017		2019		2020	
		Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% all leaf area)	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% worst affected leaf area)	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% worst affected leaf area)
1	8	65.0 a ^y	8.1 ab	38.4 a	27.1 a	26.0 a	10.8 a
2	3	25.0 b	6.0 b	38.0 a	34.5 a	25.7 a	9.5 a
3	153	62.5 a(A ^x)	8.7 ab(B)	41.1 a(B)	27.8 a(B)	30.4 a(A)	13.3 a(B)
4	171	61.6 a(A)	10.5 a(A)	47.1 a(A)	31.2 a(A)	34.1 a(A)	15.0 a(A)

^zRFLP type reflects near-isogenics groups within the tested *C. trifoliata* accessions, as designated by Fang et al. (1997).

^yWithin a column, means followed by the same lowercase letter are not statistically different at $P < 0.05$.

^xWithin a column, means followed by the same uppercase letter are not statistically different at $P < 0.05$, from a separate analysis restricted to type 3 and type 4 accessions.

Table 4. Association with chloroplast type for incidence and severity of asian citrus canker (ACC) in leaves of half-sib progenies of 84 seed source accessions exposed to field infection in Ft. Pierce, FL, for 3 years (2017, 2019, and 2020) of assessment.

Chloroplast	N, 2020	2017		2019		2020	
		Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% all leaf area)	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% worst affected leaf area)	Incidence of ACC lesions (% all leaves)	Severity of ACC lesions (% worst affected leaf area)
Pummelo	182	35.2 a ^z	8.0 a	12.8 a	11.2 a	17.3 a	11.4 a
Trifoliata	74	34.3 a	6.8 b	11.8 a	10.5 a	16.7 a	12.3 a
Grapefruit	91	34.2 a	8.8 a	15.0 a	12.1 a	16.2 b	10.6 a
Sweet orange	91	36.2 a	7.3 b	11.1 b	10.6 b	18.3 a	12.1 a
Trifoliata	74	34.3 a	6.8 b	11.8 ab	10.5 b	16.7 ab	12.3 a

^zWithin a column in each section (pummelo vs. trifoliata or grapefruit vs. sweet orange vs. trifoliata), means followed by the same letter are not statistically different at $P < 0.05$.

of the shared *C. trifoliata* accessions, and lower in severity compared with both (2019 only) of these *C. trifoliata* accessions. As noted earlier, there are two strains of *Xanthomonas* reported to be in Florida that infect citrus and produce similar symptoms in *C. trifoliata* and its hybrids (Graham et al., 1990). Infections by the strain of *X. campestris* pv. *citri* (now Xcc) were reported to be less severe in *C. trifoliata* than several other citrus types, including ‘Swingle’ citrumelo, whereas an aggressive strain of *X. campestris* pv. *citrumelo* (now Xec) was most aggressive in ‘Swingle’, followed closely by *C. trifoliata*, and was much less severe in other citrus genotypes tested. In contrast, one of the Xec strains was significantly more severe in *C. trifoliata* than in ‘Swingle’ citrumelo, which in turn showed greater lesion diameter than six of seven other citrus genotypes, and only Duncan grapefruit was similar to *C. trifoliata* in response. In another Florida field screening of ACC susceptibility, ‘Swingle’ citrumelo was reported to be the most susceptible of 10 rootstock and 8 scion genotypes, and this was adjacent to highly infected grapefruit (Futch and Graham, 2012). It seems reasonable that the greater susceptibility of *C. trifoliata* compared to its hybrids, in the trials reported here, is a result of strain differences, perhaps influenced by the planting being all *C. trifoliata* and hybrids rather than being predominately unifoliata citrus. Populations of the most aggressive strain of Xec were reported to be sustained after initial inoculation in *C. trifoliata* and hybrids, but not in other citrus types tested, in which the inoculum was undetectable at 40 d (Graham et al., 1990). Because spread and establishment of the canker pathogen is largely dependent on wind-driven rain and the stage of leaf development when

exposed, weather conditions when leaves are susceptible can have a major impact on disease development (i.e., Bock et al., 2014)

Literature support for predominance of nucellar embryony. In unpublished work, 50 seeds of each of 68 *C. trifoliata* accessions were evaluated visually for polyembryony (D. Hutchinson, personal communication), and 61 of them displayed greater than 80% of seeds as polyembryonic. Of these accessions, 24 were also included in our study and all were highly polyembryonic. Similarly, in another study, 14 *C. trifoliata* accessions were included in a larger test of citrus accessions, and 12 were predominately polyembryonic (Krueger and Roose, 2003). However, somewhat greater incidences (~30%) of zygotic embryo production are reported for some *C. trifoliata* hybrids with sweet orange and grapefruit parents (Xiang and Roose, 1988), and in crosses between 100% monoembryonic *C. maxima* × *C. trifoliata*, 92% of the progeny population produced fewer than 30% polyembryonic seeds (Kepiro and Roose, 2009).

Caution and Conclusion

Our study provides data on the relative susceptibility of *C. trifoliata* and its hybrids to ACC. Overall, hybrids of *C. trifoliata* with other citrus types displayed markedly reduced ACC sensitivity compared with *C. trifoliata*, indicating that this trait is readily overcome in breeding. For all 3 years of the study, one accession that was represented by only one tree was much less susceptible to ACC than other accessions of *C. trifoliata*, which merits further investigation. ACC incidence correlated between each pair during the 3 years these trees were evaluated. Chloroplast type

affected ACC incidence and severity, but not in a consistent manner. RFLP subgroupings for *C. trifoliata* accessions showed considerable association between RFLP type and ACC sensitivity. In Florida, there are at least three genetically different isolates of Xcc (Cubero and Graham, 2002), and likely many more around the world. Therefore, our results should, cautiously, be considered most relevant within the context of Indian River Xcc isolates. Information on relative susceptibility of diverse accessions of *C. trifoliata* and its hybrids to ACC may prove useful in breeding efforts to reduce ACC susceptibility, and may provide useful guidance for future researchers on mechanisms of resistance and susceptibility.

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