The epidemiological significance of post-packinghouse survival of *Xanthomonas citri* subsp. *citri* for dissemination of Asiatic citrus canker via infected fruit

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A B S T R A C T

The risk of introduction of *Xanthomonas citri* subsp. *citri* (*Xcc*) to new, unaffected citrus producing areas is a major concern for those citrus industries attempting to remain free of citrus canker. Citrus fruit, as a potential pathway for *Xcc* to enter and become established in these areas, are assumed to be a risk. However, there is little information relative to the potential of harvested fruit to act as an inoculum source. A multi-national research team was established to investigate the potential of bacterial survival in infected citrus fruit lesions and as surface contaminants on symptom-free fruit, and to examine the potential of infected fruit as a viable inoculum source. Experiments were conducted in various locations in Florida and Argentina. Bacterial recovery and culture plating were problematic due to the presence of non-pathogenic bacteria with cultural characteristics that were difficult to distinguish from *Xcc*. Therefore, in all experiments, although culturing on semi-selective agar media was used as an indication of overall bacterial populations, bioassays were conducted via needleless injection and infiltration of suspect bacterial suspensions into susceptible cv. Duncan grapefruit leaves. Inoculation sites were subsequently assessed for symptoms of citrus canker and lesions were individually enumerated to confirm the presence of *Xcc*. In commercial packing lines in Florida and northwest Argentina, prewashing the fruit to remove dirt and debris reduced surface bacterial populations. As anticipated, recovery of *Xcc* from fruit surfaces increased when active citrus canker lesions were present but total bacterial recovery decreased after processing, and bioassays demonstrated that the quantity of viable *Xcc* declined as fruit remained in cold storage, or as they aged on the trees. Bioassays demonstrated that the highest incidence of *Xcc* from fruit after the packing line antimicrobial treatment occurred with symptomatic fruit (2.5–50.6 lesions leaf⁻¹), and zero to very low levels with fruit from apparently healthy trees (0–1.74 lesions leaf⁻¹). Furthermore, the proportion of injection–infiltration bioassay sites that developed lesions consistently decreased with time after processing in each of the three packinghouse studies, also showing that as fruit senesce and lesions age the ability of fruit to generate or sustain *Xcc* bacteria was increasingly compromised. The packing line process reduced canker lesion activity by as much as 50% compared to unprocessed fruit. *Xcc* survived in wounds on mature fruit attached to the tree, but *Xcc* populations declined in wounds of processed or non-processed harvested fruit. Discarded canker-infected fruit in cull piles was ineffective as a source of inoculum for dispersal. Transmission from cull piles of packing line-processed fruit to surrounding trap plants, even less than 1 m away, did not occur under natural conditions. However, with severely infected piles of culled fruit subject to extreme simulated wind conditions.

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1. Introduction

Asiatic citrus canker is a foliage, stem, and fruit spotting disease of citrus caused by the bacterial pathogen Xanthomomas citri subsp. citri (Xcc) (Syn. Xanthomomas axonopodis pv. citri). In subtropical areas of the world where citrus canker mostly occurs, the disease is characterized by erumpent lesions on fruit, foliage, and young stems of susceptible cultivars of citrus, especially grapefruit. When citrus canker is severe, defoliation, dieback and fruit drop can occur and remaining infected fruit have limited or no marketability (Gottwald et al., 2002; Schubert et al., 2001; Graham and Gottwald, 1991; Koizumi, 1985). Bacteria exude from lesions when they are wetted by dew, rain, or irrigation. This bacterial inoculum is easily splash dispersed by rain and this process is greatly facilitated by wind (Bock et al., 2005), resulting in spread over potentially long distances (Gottwald and Irey, 2007). When rain is combined with wind speeds in excess of 8 m s$^{-1}$, numerous new infections from rain-borne inoculum results in disease that can be severe (Serizawa and Inoue, 1974; Gottwald et al., 2002; Gottwald and Irey, 2007).

Survival of Xcc within lesions on fruit or on fruit surfaces is key to fruit acting as a pathway for dissemination of inoculum. Xcc can survive in high numbers within foliar, stem, and green fruit lesions (Shirotani et al., 2009; Gottwald et al., 2002; Schubert et al., 2001), whereas, outside plant tissues, the bacterium apparently has very limited survivability. Assessment of Xcc populations on artificially-infested surfaces of metal, plastics, cloth, and processed wood in both shade and sun indicated that the inoculum was no longer culturable after 24–72 h (Graham et al., 2000). In addition, the ability of the bacteria to be dispersed from leaf lesions has been studied (Bock et al., 2005), as has infection of plants in wind-driven spray (Bock et al., 2006). In contrast, the epidemiological potential of harvested fruit to produce inoculum and act as sources of infection has only recently been considered in a single study on Satsuma mandarin from Japan (Shirotani et al., 2009). These authors found no evidence for prolonged survival of bacteria on healthy fruit surfaces or on discarded fruit within an orchard. Furthermore, they could find no spread from mature, asymptomatic but Xcc-contaminated fruit of Satsuma mandarin placed in a citrus orchard. These results, while indicative, are not necessarily directly comparable to the situations in Florida, Argentina or Brazil, where different hosts and environmental factors are involved and where there are additional concerns regarding survival and spread of Xcc from infected or contaminated fruit both pre- and post-packing procedures. Further studies are thus needed to corroborate the observations from Japan and also respond to additional issues regarding fruit as a potential pathway.

It is salient to define the terms being used to describe the disease status of a citrus fruit. This will help avoid confusion or misunderstanding and in this study we define the terms used as follows:

1. Symptomatic fruit – a fruit showing obvious symptoms of citrus canker infection.
2. Asymptomatic fruit – fruit that is known to be infected or contaminated with Xcc, but does not show any symptoms.
3. Apparently healthy fruit – a fruit that has no evident symptoms of citrus canker and is assumed to be disease-free, but conceivably might still be contaminated or infected.

To prevent the introduction of Xcc, many citrus-growing areas restrict the importation of citrus from areas or countries known to have endemic citrus canker. The best approach is pathogen exclusion to maintain canker-free areas and avoid loss of markets. Various regulatory risk analyses have been conducted to evaluate the potential for fruit to act as a pathway for Xcc to be transported and become established in unaffected citrus producing areas (Anon., 2007). These analyses are based on existing, published data for Xcc spread, transmission and disease establishment. However, critical information gaps remain, that if bridged, would more clearly establish potential risk due to these sources (Shirotani et al., 2009). However, in areas where Xcc is endemic, the challenge is to manage the disease to reduce or avoid crop losses and to develop a systems approach for production of healthy fruit and packinghouse inspection procedures to eliminate asymptomatic and asymptomatic fruit has limited effectiveness when fruit disease incidence exceeds 2–5%. This has led some citrus producing countries, such as Argentina, to attempt to maintain “canker-free” zones. In Argentina the northwestern Provinces of Salta, Jujuy, and Tucumán were previously maintained as canker-free zones for export purposes (Hogg, 1985), although the disease is currently established in many parts of this region as well.

Present USDA regulations have a zero tolerance for canker lesions in packed boxes of fresh fruit (APHIS–USDA, 1997; Anon., 2007). Evaluation of the risk to healthy plants of infection from discarded, fruit with active citrus canker lesions will provide a scientific understanding on which to base the regulation of trade of fruit either contaminated with, or infected by Xcc. If a significant level of risk exists, more rigorous inspections in packinghouses, combined with careful practices for disposal of fruit might be necessary. Therefore, this study included a series of experiments designed to address several knowledge gaps of the potential risk of infection to healthy plants from discarded, cankered fruit. The overall objective was to determine the potential for infected or contaminated fruit to transport, or be a source of, viable Xcc inoculum, and for that inoculum to cause infection in distant, healthy citrus trees, resulting in spread of disease to hitherto canker-free citrus producing regions. Firstly, we examined the effectiveness of current and modified packinghouse decontamination measures to reduce the recovery of Xcc from infected and infected fruit. Secondly, we examined the epidemiological potential for citrus fruit with lesions that have passed through the packinghouse undetected to act as a source of inoculum for establishment of disease on healthy plants in the field. Thirdly, we assessed the risk of infection from unprocessed, discarded cankered fruit under simulated severe wind–rain conditions. Because of national and international market implications, experiments were conducted in both Florida and Argentina. Researchers from US citrus producing states as well as citrus production areas in Argentina, Brazil, and the EU participated in various aspects of the study including experimental design, study execution, data analysis, and interpretation.
2. Materials and methods

Because individual research studies were conducted and/or repeated in various locations in countries with different fruit cultivars as well as different packinghouse designs, there were variations in experimental design. However, individual experiments were designed to yield comparable results among analogous objectives.

The studies addressed three critical questions relative to Xcc survival. In the first set of experiments, survival of Xcc in lesions and on fruit surfaces prior to and after packing line decontamination and the subsequent survival of the bacteria in and on processed fruit after storage for various time periods was examined. The second group of experiments dealt with survival of bacteria in wounds that might occur during harvest, in the packinghouse, or post-packinghouse that might promote the persistence of bacterial populations. The third group of experiments was designed to examine the potential of Xcc-infected fruit to act as a source of infection via wind and rain for transmission to susceptible citrus hosts in proximity to the inoculum.

2.1. Determining the survival of Xcc on fruit before and after packing line processing

Experiments in Florida used grapefruit and in Argentina used lemon. Prewash trials were conducted to determine the best means of decontamination of fruit surfaces, and cold storage treatments examined survival of bacteria and Xcc post-packing line treatment through time.

2.1.1. Grapefruit prewash trial

In one study, prewashing fruit with disinfectants significantly reduced Xcc bacteria to non-detectable levels (Canteros et al., 2000). To determine the effect of prewashing fruit on bacterial survival on apparently healthy cv. Ruby Red grapefruit (Citrus x paradisi Macf.), fruit were harvested from canker Xcc-infected trees in an orchard located in Ft. Pierce, FL on 11 January 2007. Three replications of five fruit were harvested for each treatment. Fruit were treated as follows: (1) an untreated control, (2) a chlorine immersion (200 µl l⁻¹ at pH 7.0), (3) a prewash with water followed by chlorine immersion, and (4) a prewash with water plus detergent (Fruit Wash 395, FMC Foodtech, Lakeland, FL) followed by chlorine immersion. The water and detergent washes were each performed for 45 s on rotating, soft bristled brushes with an overhead sprayer. Immersion in chlorine solution was for 45 s. Fruit received treatment the day after harvest and were stored at ambient temperature in the packinghouse.

Five replicate fruit from each treatment were assayed immediately following treatment for total surface bacteria by placing each in a separate sealable plastic bag containing 100 ml of sterile phosphate buffered saline (PBS; 0.075 M; pH 7.0) and sonicated for 5 min to loosen and release surface bacteria. The wash solution was centrifuged at 10,000 g for 15 min. The resultant pellet was resuspended in 5 ml sterile PBS and the suspension was diluted to 10⁻¹ and 10⁻² and 100 µl spread on triplicate plates of KCB medium (nutrient agar plus kasugamycin 16.0 mg l⁻¹, cephalexin 16.0 mg l⁻¹, and chlorothalonil 12.0 mg l⁻¹) (Graham and Leite, 2004). Plates were incubated at 28 °C for 3–7 d. Total bacterial colonies per plate were counted and expressed as cfu ml⁻¹.

2.1.2. Lemon prewash trial

To determine the effect of prewashing on Xcc survival on apparently healthy lemons (Citrus limon (L.) Burm. f.) cv. Lisbon, apparently healthy fruit were harvested from infected trees in an affected orchard and the unsprayed check from a chemical control trial in another orchard. Both orchards were located in Tucumán Province, Argentina. Prior to fruit harvest, the orchards received several rain showers with occasional wind speeds exceeding 8 m s⁻¹ and disease was widespread. Three replications of five fruit were harvested for each of the following treatments: (1) an untreated control, (2) a chlorine wash (200 µl ml⁻¹ at pH 7.0) by immersion for 2 min, (3) chlorine immersion for 2 min followed by a detergent spray (Neutro Deter N, Sinner S.A., Tucumán, Argentina, 2%) for 20 s, (4) a prewash with water followed by chlorine for 2 min, and (5) prewash with water followed by chlorine for 2 min followed by detergent for 20 s.

To determine the population of Xcc on fruit, the wash solution was assayed by injection–infiltration of the suspension into two immature leaves on greenhouse grown potted cv. Duncan grapefruit seedlings as previously described (Graham and Leite, 2004). One ml of suspension was infiltrated with a needleless syringe into eight sites on the abaxial surface of each leaf. The seedlings were returned to the greenhouse (diurnal temperature 26–28 °C and RH 80–90%) and the inoculated foliage covered with a plastic bag for 48 h. Total number of lesions per leaf were counted 14 d after inoculation from all injection sites.

2.1.3. Packing line experiment for grapefruit

Fruit were harvested on 10 April in 2006 and 16 April in 2007 from a canker-affected orchard of cv. Ruby Red grapefruit in Ft. Pierce, Florida. Prior to packinghouse processing, the harvested fruit were placed in four treatment groups of 200 fruit each, depending on their symptomology and proximity to sources of inoculum in the orchard. The day after harvest, fruit were divided into the following treatments: (1) apparently healthy fruit from apparently healthy trees (no visible canker lesions, but trees located in close proximity to infected trees, i.e., <30 m), (2) apparently healthy fruit from infected trees in the orchard, (3) symptomatic fruit from infected trees, and (4) a combination of symptomatic fruit from infected trees and apparently healthy fruit from healthy trees at a ratio of 1:4, symptomatic:apparently healthy, respectively. Symptomatic fruit were defined as having 1–5 lesions. In the combination treatment, symptomatic and apparently healthy fruit were recovered from the mixture and assayed separately.

Fruit were processed on the packing line at the University of Florida, Indian River Research and Education Center (IRREC), Fort Pierce, Florida (Allen, 2003). In the 2006 experiment, the fruit were initially immersed in a solution of chlorine (200 µl ml⁻¹) for 45 s followed by detergent (Fruit Wash 395 (sodium-ortho-phenylphenate, 2% SOPP), JBT Foodtech, Lakeland, Florida, USA) for 30–45 s on rotating soft bristled brushes, followed by a water rinse spray for 45 s and spray application of a shellac-based wax (FMC Ata-Fresh 590HC, JBT Foodtech, Lakeland, Florida, USA) plus imazalil fungicide 2000 µg g⁻¹ (FMC Freshguard 700 and Imazalil 44.6 EC, JBT Foodtech, Lakeland, Florida, USA) on rotating brushes for 45 s (Allen, 2003). In 2007 a 45 s prewash with detergent was followed by the chlorine immersion for 45 s, then an SOPP spray for 45 s on rotating brushes, followed by a water rinse (rinse was not recirculated) and application of a carnuba-based wax (FMC Sta-Fresh 2109, JBT Foodtech, Lakeland, Florida, USA) plus imazalil (2%). In both 2006 and 2007, 1 d after harvest, three replicate samples of five fruits from each of the four treatments were assayed to determine the pre-treatment levels of Xcc and total bacteria on fruit. After processing fruit were placed in storage (approximately 50% relative humidity) at 5–8 °C, and samples of three replicate groups of five fruits were taken on d 1, 4, and 7 in 2006 and 2007, and d 21 in 2007 only. The fruit from each sample were biossayed for surviving Xcc by injection–infiltration of the suspension into two immature leaves of potted cv. Duncan grapefruit seedlings as described for the lemon prewash trial with the following
modifications: seedlings were placed in a greenhouse (21–27 °C and RH 50–60%) and the total number of lesions per leaf counted 14 d after inoculation. In 2006 recovery of bacteria was made as described above for the grapefruit prewash trial, and the resulting suspension plated onto KCB medium using a spiral plater (Spiral Biotech, Inc., Bethesda, MD), and the total number of bacterial colonies counted using a Q-Count colony counter (Q-Count, Advanced Instruments Inc., Norwood, MA). In 2007, recovery of bacteria was made as for 2006, but the resulting suspension was direct plated onto KCB medium in a dilution series, and colony numbers counted visually.

2.2. Survival of Xcc in fruit wounds

Repeated 1, 4 and 7 d post-processing. 100 Ruby Red were artificially wounded by needle prick, or a 1-cm cut on fruit surfaces under pre-harvest orchard and packinghouse conditions. The inoculum was prepared by excising 50 canker lesions from cv. Ruby Red grapefruit leaves and grinding them in PBS. The suspension was filtered through a sterile gauze pad, and PBS was added to bring the final volume of inoculum to 50 ml before inoculation. The inoculum was prepared by excising 50 canker lesions from cv. Ruby Red grapefruit leaves and grinding them in PBS. The suspension was filtered through a sterile gauze pad, and PBS was added to bring the final volume of inoculum to 50 ml before inoculation. The inoculum was prepared by excising 50 canker lesions from cv. Ruby Red grapefruit leaves and grinding them in PBS. The suspension was filtered through a sterile gauze pad, and PBS was added to bring the final volume of inoculum to 50 ml before inoculation. The inoculum was prepared by excising 50 canker lesions from cv. Ruby Red grapefruit leaves and grinding them in PBS. The suspension was filtered through a sterile gauze pad, and PBS was added to bring the final volume of inoculum to 50 ml before inoculation.

2.3. Dispersal of Xcc from fruit in discarded cull piles

Experiments at four locations in 2006–07 tested the potential of cull piles to serve as sources of inoculum for Xcc dispersal. Three cull pile tests were conducted in Florida, and one in Argentina. These experiments relied on natural and simulated wind and rain events to assess dispersal of bacteria of Xcc.

2.3.1. Ft. Pierce Florida fruit cull pile experiments

The symptomatic fruit used as a source of inoculum were post-packing line-processed fruit from the packing line study, originally collected from an infected cv. Ruby Red grapefruit orchard near Fort Pierce, Florida. The cull pile was placed in the field immediately after completion of the packing line study. The cull pile experiment was repeated once. In the first experiment, eight disease-free cv. Duncan grapefruit trees were transplanted from 3.8 l containers into the ground as trap plants placed equidistant from each other in each of two concentric circles, at 1 and 10 m from the inoculum source (total of 16 trees). Infected fruit with one to multiple lesions were placed in the field in a cull pile configuration on 5 June 2006, and the experiment ended 21 July 2006. The location was in an open field at the USDA farm in Ft. Pierce, the topography flat and there were no windbreaks within 200 m. The repeat experiment was begun on 26 April 2007, and terminated 18 June 2007, and included a third concentric circle 5 m from the inoculum source. Each circle again contained eight cv. Duncan grapefruit trees spaced equidistant around the circle. In both experiments the plants were inspected for symptoms of Xcc infection on a weekly basis.

2.3.2. Gainesville, Florida fruit cull pile experiments

Commercially graded grapefruit with citrus canker lesions were selected at various times throughout 2007. The infected fruit were processed on commercial packing lines, and were placed in the field within 1–3 weeks of processing. Prior to placement in the field they were stored under ambient room conditions (23 °C). Selected fruit had from one to multiple lesions. Disease-free cv. Duncan grapefruit seedlings were planted in 10-cm diameter containers and
embedded in the ground in a simulated orchard setting under two different regimes; the first having fruit with a single canker lesion and the second having fruit with multiple lesions (~100). Single lesion fruit were placed with the blemish up to ensure maximum chance for splash dispersal. The multiple lesion fruit were included to see if fruit with severe infections were a better source of inoculum. Four healthy cv. Duncan grapefruit seedlings were placed around each fruit set at a distance of 0.15 m. Within each set of experiments (single lesion vs. multiple lesions) there were four replications. Experiment 1 was conducted 5 January to 28 March 2007 (plants were monitored for disease until 20 June); Experiment 2 was conducted 1 May to 21 June 2007 (plants were monitored for disease until 22 August); Experiment 3 was conducted 3 July to 23 August 2007 (plants were monitored for disease until 5 October); and Experiment 4 was conducted 5 October to 4 December 2007 (plants were monitored for disease until 25 January 2008). The trap plants in containers were transferred to an enclosure for incubation. The infected fruit surface and plant leaves were assayed for Xcc following dew (periods when surface moisture was seen on the leaf surface) and rain events by swabbing the wet surfaces with sterile cotton-tipped applicators then streaking the swab tip on the surface of KCB medium. The applicators were placed in 1 ml PBS and incubated for 15 min. The suspension was plated on KCB and incubated as described above and assayed for Xcc colonies. The remaining suspension was bioassayed by injection–infiltration into young leaves of cv. Duncan grapefruit. At the conclusion of the experiment a lesion was excised from the peel and placed in an Agdia (Agdia Inc., Elkhart, IN) sample extract bag with 1 ml of PBS, macerated using an electric press and 100 µl of dilutions from 10^4 to 10^-4 were plated on KCB medium. A bioassay was done as described above. The grapefruit seedlings used for bioassay were observed for canker symptoms for 3–4 weeks.

2.3.3. Tucumán, Argentina fruit cull pile experiments

Field collected untreated, lemons with canker lesions were used as the source of inoculum. Disease-free cv. Duncan grapefruit seedlings (35–40 cm tall) with two to three shoots of susceptible tissue, were used as trap plants and placed equidistant in concentric circles 1, 5, and 10 m from the cull pile of infected fruit. There were a total of 8, 8 and 16 trees in each circle, with interplant distances of 0.78, 3.8 and 3.8 m within a circle, respectively. Both the cull pile and the trap plants were replaced each month from October 2006 to November 2007. The grapefruit seedlings were monitored on a monthly basis for symptoms of citrus canker. Plants were kept for an additional 2 weeks following the conclusion of the field experiment to check for post-experiment lesion development. Leaves were washed and plated on KCB medium as described above, and a portion of the wash was bioassayed on grapefruit as previously described. Colonies that grew on KCB medium and appeared to be Xcc were also bioassayed on cv. Duncan grapefruit indicator plants. In addition, suspicious colonies on KCB were also tested using indirect immuno-fluorescence (IF) with a polyclonal antibody (Brlansky and Lee, 1990).

2.3.4. Simulated bacterial dispersal from fruit cull piles and suspended fruit

A third set of experiments was conducted in South Florida in 2007 to determine whether bacteria dispersal from mature, infected fruit and infection of susceptible citrus was possible under extreme conditions of wind and rain. To test this in the field, an airboat fan and motor affixed to a trailer were used to simulate wind in two experiments, each repeated four times. The airboat fan (airplane propeller) can produce wind in excess of 27 m s⁻¹ (96 km h⁻¹), generated by a 5735 cm³ (350 in³), high performance, 300 hp, 8-cylinder gasoline engine (Chevrolet, General Motors, Detroit, MI, USA). Two experimental designs were used, one that had as the inoculum source a cull pile of infected fruit (Fig. 1A), the other columns of suspended fruit on six vertical strings in a ~1.0-m² wooden frame (Fig. 1B). All the infected grapefruit were collected from a citrus canker-affected orchard, near Ft. Pierce, Florida and were severely infected, but had not been processed through a packing line. Each cull pile and suspended fruit set was used for two replicates of each experiment, making a total of four repetitions for each of the experiments. There were 105 and 116 fruit in the first and second cull pile experiments, respectively. In the suspended fruit experiments, there were 27 and 39 fruit in the first and second, respectively. The airboat fan was set to produce wind of 0, 10 and 25 m s⁻¹, and rain was simulated using garden sprayers and a non-chlorinated water source that dispensed water into the air-stream. Wind speed and temperature were monitored throughout the experiments using cup-anemometers and temperature sensors (Campbell Scientific, Logan, UT). In the cull pile experiment, fruit were placed as a pile on a raised surface (65 cm above ground level, 50 × 80 cm area) in line with the airflow. In the suspended fruit experiment, fruit were suspended on strings across the airflow (suspended between 1 and 2 m above ground). In both experiments, fruit were 4 m downwind from the fan, and panel samplers utilized to collect the splash (Parker et al., 2005) were set up at 0.2, 5 and 10 m distant from the cull pile. The collection panels (30 × 15 cm) were placed at a height of 65 and 140 cm for the cull pile experiments. Four cv. Duncan grapefruit seedlings acted as trap plants at each distance at the same height as the lower panel. The seedlings were approximately 25 cm tall and were chosen while in flush with new leaves approximately 3/4 -expanded. For the suspended fruit experiment a single panel at 65 cm height was set at each distance along with four trap plants. At the end of each treatment run, panels were washed with 50 ml water prior to plating on KCB medium. The simulated rain was introduced into the airflow upwind of the culled fruit so it fell on the pile of fruit. Each wind speed treatment was run for 5 min. The spray and splash collected by the panels were plated on KCB media as previously described and suspect colonies identified using the immunostrips designed for detection of Xcc (Xcc ImmunoStrip™ Test, Agdia, Inc., Elkhart, IN). Trap plants were incubated in a greenhouse and assessed for infection after 3 weeks. Bacteria production was tested by washing each of five fruit in 10 ml sterile distilled water for 1 min and plating 0.1 ml aliquots of each of a dilution series, then testing individual colonies with the immunostrips.

2.4. Dispersal from infected citrus peels

Citrus peel from an infected fruit that might be discarded in the vicinity of a healthy tree was used to test the ability of infected citrus peel to act as an inoculum source. Citrus fruit peel with lesions of Xcc (from fruit collected in the same planting as for the cull pile experiments) was removed from several infected cv. Ruby Red grapefruit. Four sections of peel were removed uniformly from each fruit as 1⁄4-grapefruit peel pieces. Lesion size on these sections ranged from 0.1 to 0.5 cm. The peel was placed in fiberglass mesh storage bins to protect it from scavengers. One bin was placed in the shade under a grapefruit tree, and the other out in the open and exposed to the sun, but adjacent to the first, at a citrus planting in east-central Florida. The peel lesions were sampled arbitrarily from the population of peel sections and lesions in the mesh bags on a weekly basis to test for viability of canker bacteria in lesions. On the first sampling, five lesions were sampled, and on all subsequent occasions 10 lesions were sampled and crushed in a microfuge tube in 1 ml sterile PBS and the resulting suspension plated onto KCB medium and bioassayed as before. This experiment was not repeated.
2.5. *Xcc dispersal from burst infected fruit*

The final experiment was designed to address a proposed scenario for potential dissemination of *Xcc* from cankered fruit by, for example, a child who might choose to hit fruit with a baseball bat, thereby driving potential infected fruit debris into a susceptible citrus tree. All fruit were collected from a cv. Ruby Red grapefruit orchard near Ft. Pierce, Florida. Grapefruit were placed individually on top of a PVC tube 1 m above ground level and 4 m from a rack containing 10 cv. Duncan grapefruit seedlings (0.5 m tall, approximately 1 year old) in 4 l containers. Infected fruit were then impacted with a baseball bat, and propelled toward the grapefruit trap plants. There were three fruit types—apparently healthy fruit from apparently healthy trees, apparently healthy fruit from symptomatic trees, and symptomatic fruit from symptomatic trees. The experiment was repeated once. In the first run, four individuals batted 10 grapefruit of each treatment so that they burst and splattered the flesh and juice over the 10 grapefruit seedlings. This was done for each treatment starting with the apparently healthy fruit from apparently healthy trees, then the apparently healthy fruit from symptomatic trees, and finally the symptomatic fruit from symptomatic trees. There were 10 grapefruit seedlings per replicate with four replicates (40 plants total) in the first experiment, and six replicates in the second (60 plants total). In the second run, five individuals served as batters. Again, the trees were separated by treatment to avoid contamination. Subsequent to each experiment, the trap plants splattered with fruit debris and juice were placed in a greenhouse at 25–30 °C and incubated for 4 weeks and checked for development of citrus canker symptoms. A single fruit from each treatment was selected and placed in a plastic bag.
with 100 ml PBS, sonicated for 5 min and then plated and bio-
assayed as previously described. Leaf washes were taken from
exposed plants in the repeat run only (10 leaves treatment−1) prior
to incubation and plated on KCB medium using a spiral plater as
described above.

2.6. Data analysis

The bioassay data (lesion counts) and bacterial population data
(cfu ml−1) from the prewash trail in Florida and Argentina and the
three packinghouse studies were analyzed by general linear
modelling (Proc GLM) using SAS (SAS systems, Cary, NC). Data were
log transformed prior to analysis due to heterogeneity of variance
in all the groups of fruit source and time tested. The main effect
means of each fruit treatment, storage time or harvest date were
calculated and a means separation for the various main effects and
interactions calculated using Tukey’s HSD test. For the bioassay
tests, the proportion of sites that developed lesions was also
calculated. However, because the proportion was non-replicated it
was not amenable to analysis, but is presented as it provides useful
additional information on viability of the canker bacteria. Data from
the 2006 study on survival of Xcc in fruit wounds were analyzed with
regression analysis. Power law models were fit to the data to
describe the relationship between surviving numbers of Xcc bacteria and time for each treatment group of fruit. Data from the
2007 trial were more variable with time, which precluded a meaningful regression analysis, but are described nonetheless.

3. Results

3.1. Survival of Xcc on fruit before and after packing line processing

3.1.1. Grapefruit prewash trial

Recoverable bacteria varied among treatments (F = 119.5,
Pr > F < 0.0001) based on GLM analysis. The chlorine treatment
alone did not reduce total bacterial (Xcc plus other bacteria) recovery compared to the control (Fig. 2A) based on Tukey’s means
separations. Prewashing fruit and treating with chlorine signific-
antly reduced total bacterial recovery compared to the control of
chlorine treatment alone, and prewashing fruit followed by chlo-
rine and detergent was the most effective method for killing bacteria on the fruit surface.

3.1.2. Lemon prewash trial

All treatments were in the same grouping based on Tukey’s
means separation (Fig. 2B), but the bioassay lesion counts were low
(maximum of 3) and the frequency of zero lesions relatively high
across all treatments. There were no significant differences among
the treatments (F = 0.70, Pr > F = 0.59) based on GLM analysis.
Nonetheless, there was a trend suggesting that chlorine treatment
slightly reduced the number of lesions recovered, and chlorine
treatment after prewashing the fruit, with or without detergent was
beneficial in reducing the number of Xcc recovered from the fruit.
Thus, the Florida and Argentina studies suggest that effectiveness of
packing line decontamination can be increased by using prewash-
ting treatment that includes detergent (such as SOPP) to remove dirt
and debris that reduce the effectiveness of the disinfectants.

3.1.3. Packing line experiment for grapefruit

In 2006, washates of symptomatic fruit from an infected tree produced the highest number of canker lesions (F = 4.32,
Pr > F = 0.0063) in the bioassay for viable Xcc, while all other fruit
sources, including apparently healthy fruit from an apparently
healthy tree, the least (Fig. 3A). The number of lesions in the
bioassay of washates of fruit sampled preprocessing and after cold
storage was significantly different (F = 3.16, Pr > F = 0.0273).
However, the means separation test used (Tukey’s) did not
demonstrate this, although preprocessed fruit washates produced
greatest numbers of Xcc lesions (Fig. 3B). Data were highly variable
(e.g., no viable bacteria were detected in washates of several fruit in
all treatments, and large numbers of bacteria were detected only in
washates of a few fruit). The greater population of Xcc in this
sample on d 7 in cold storage could be accounted for by two fruit
that had lesions with high viable Xcc populations and produced
~1000 lesions in the bioassay. However, the proportion of bioassay
inoculation sites that produced bacteria declined with time
(Fig. 3C), suggesting that the potential of lesions on fruit overall
to generate Xcc declined with time. Initially 0.53% of injection infil-
tration sites developed lesions, but by d 7 this decreased to only
0.11% of sites. Symptomatic fruit from an infected tree had the
highest population of total bacteria (F = 27.32, Pr > F < 0.0001) and
asymptomatic fruit mixed with symptomatic fruit, and
apparently healthy fruit from an apparently healthy tree the lowest
(Fig. 3D). Fruit sampled prior to processing had significantly higher
total bacterial populations compared to fruit sampled 1 d after
processing (F = 4.08, Pr > F = 0.0086, Fig. 3E). Numbers of bacteria
increased after d 1 in cold storage; most likely due to an increase in
population of surface bacteria on the fruit subsequent to
processing. On d 7, two fruit each produced ~20,000 total bacterial ml−1. The symptomatic and apparently healthy fruit in the
combined sample were processed separately and very few bacteria
were retrieved from the apparently healthy fruit (n = 0.03 ml−1),
while 34 bacteria ml−1 were found on the symptomatic fruit.
Similarly, no infection developed with washates from the symp-
tomatic fruit in the bioassay, but a mean of 52 lesions leaf−1
developed with washates from the symptomatic fruit, suggesting
the risk of contamination from symptomatic fruit was exceedingly
low.

In 2007, the bioassay for Xcc showed a significant effect of fruit
source on recoverable Xcc (F = 3.22, Pr > F < 0.0272), although
the means separation using Tukey’s did not reflect this (Fig. 4A),
washes from the symptomatic fruit from an infected tree
produced the most lesions (0.19 lesions leaf−1), while apparently
healthy fruit from an apparently healthy tree had no recoverable
Xcc. Interestingly, and in contrast to 2006, the bioassay demon-
strated no significant effect of days in cold storage (F = 0.95,
Pr > F = 0.4407) on the number of lesions. The number of lesions
developing in bioassay from washates of fruit sampled prior to
processing was not significantly greater than any other day post-
processing, despite a general decline in number of lesions in
bioassays with time, and no canker lesions developed after 21 d of
cold storage (Fig. 4B). The lack of statistical confirmation of this
trend is likely due to high variance caused the disparity between
washates of several fruit in all treatments that resulted in no
bacterial recovery and recovery of high bacterial populations from
a few fruit. Recovery from a few fruit with high bacterial popu-
lations was reinforced by the data on the proportion of individual
bioassay tests that produced a positive result (Fig. 4C). Pre-packing
line processing 0.14% of injection infiltration sites produced lesions,
but by d 21 no lesions developed in any site. Fruit source had no
effect on total population of bacteria (F = 0.41, Pr > F = 0.7448) and
all treatments had recoverable bacteria (Fig. 4D). However, there
was an effect of days in cold storage on bacteria populations
(F = 29.42, Pr > F < 0.0001) based on the GLM analysis. Fruit
sampled prior to processing, and on d 21 in cold storage had
significantly higher bacterial populations compared to post-pro-
cessed fruit on other days (Fig. 4E), showing a similar trend to the
data from 2006 and most likely due to an increase in residual
populations of general surface bacteria (non-Xcc) on the fruit in
cold storage subsequent to the processing. As in 2006, the
symptomatic and apparently healthy fruit in the combined sample were processed separately; however, this time the population of total bacteria on healthy fruit (3.17 × 10^4 ml⁻¹) was similar to that for the symptomatic fruit (2.43 × 10^4 ml⁻¹). Nevertheless, the washates of the apparently healthy fruit produced no lesions of canker in susceptible leaves of grapefruit, while washates of the symptomatic fruit produced 10 lesions leaf⁻¹, confirming the results of the 2006 trial.

3.1.4. Packing line experiment for lemon

The source of the fruit affected the number of lesions detected via bioassay (*F* = 15.29, *Pr > F* = 0.0001), with most lesions developing from symptomatic fruit taken from an infected tree, and less from the other fruit source types (Fig. 5A). The number of canker lesions developing on leaves was greatest pre-packing line processing and least on d 7 in cold storage in bioassays using washates of fruit (*F* = 4.75, *Pr > F* = < 0.0030, Fig. 5B). The proportion of bioassay injection–infiltration sites that produced bacteria also showed a pronounced decline with time (Fig. 5C), consistent with the results from the 2006 and 2007 Florida trial. No viable *Xcc* was detected by bioassays of washates of fruit harvested in June or August after d 7 in cold storage. The injection–infiltration bioassay showed that processing fruit through a packing line reduced the activity of canker lesions by approximately 50%, as shown by the number of recoverable *Xcc* bacteria from packing line treated vs. non-packing line treated samples (*F* = 9.62, *Pr > F* = < 0.0021, Fig. 5D) and inoculum declined (*F* = 27.03, *Pr > F* = < 0.0001) depending upon the month of sampling, with more lesions caused by *Xcc* occurring in fruit harvested in April, and fewest later in the season in August (Fig. 5E).

The source of the fruit also affected the bacterial population (*F* = 43.05, *Pr > F* = < 0.0001), with most bacteria being isolated from symptomatic fruit from an infected tree, and none being isolated from apparently healthy fruit from an apparently healthy tree (Fig. 6A). The number of bacteria isolated from the fruit was affected by the days kept in cold storage (*F* = 4.77, *Pr > F* = < 0.0030). The greatest quantity of bacteria was isolated on d 1, and the quantity declined thereafter to d 7 (Fig. 6B). Fruit processing affected the population of bacteria (*F* = 23.44, *Pr > F* = < 0.0001), with the pre-processed sample having substantially more bacteria (Fig. 6C). Furthermore, the number of bacteria isolated (*F* = 14.88, *Pr > F* = < 0.0001) was profoundly affected by the month of harvest, with most bacteria being isolated from the fruit surface in April, and fewest later in the season in August (Fig. 6D).

3.1.5. Combined Florida and Argentina packing line outcomes

This reproducible reduction in *Xcc* viability from fruit lesions, is corroborated by the concomitant general decline in number of lesions per leaf produced by the samples from Argentina and Florida over time. A comparison of packing line treated vs. non-treated samples in Argentina (Fig. 5D) also demonstrated packing line processing per se reduced activity of canker lesions by approximately 50%. Samples that had not passed through the packing line had more active lesions, which suggest that even if infected fruit passes through a packing line, the ability of the lesions to produce bacteria had been significantly reduced.

Furthermore, in Argentina, many fewer *Xcc* bacteria were reisolated from naturally-occurring fruit lesions in August compared to April, reflecting the effect of fruit age (and therefore lesion age) on inoculum production. Although not always significantly different,

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Fig. 2. Effects of citrus fruit prewash treatments on the population of total bacteria from (A) apparently healthy cv. Ruby Red grapefruit and mean number of lesions resulting from bioassay of washates of (B) cv. Lisbon lemon. The grapefruit and lemons were harvested from canker-affected trees in orchards located in Ft. Pierce, Florida and Tucumán Argentina, respectively. Grapefruit were untreated (control), immersed in chlorine (200 μl ml⁻¹), prewashed with water followed by immersion in chlorine, or prewashed with water plus detergent followed by chlorine. Lemons were untreated (control), immersed in chlorine (200 μl ml⁻¹), immersed in chlorine plus detergent, prewashed with water followed by chlorine, or prewashed with water plus detergent followed by chlorine. *Xanthomonas citri subsp. citri* (*Xcc*) on lemon fruit was assayed as number of lesions produced after the washate was bioassayed by injection–infiltration of bacteria into replicate leaves of cv. Duncan grapefruit seedlings. Total bacterial cfu ml⁻¹ recovered from the washed fruit are the mean of three groups of five fruit. Bars with the same letters are not significantly different from each other based on Tukey's means separation.
the numbers of lesions produced in bioassays by samples taken from fruit in cold storage tended to decline with time. Some of these data were variable among all groups, and it is pertinent to note that an occasional lesion may remain viable, with infective Xcc and result in a spike in infection as noted on d 7 in the 2006 Florida experiment. By d 21 in 2007, no lesions were produced in the bioassays of washates from packinghouse fruit, the only experiment that checked inoculum production from lesions over such a long period. Thus, it appeared that Xcc population levels generally declined in fruit lesions up to d 7 compared to pre-treatment levels, and in one experiment ceased to produce detectable, viable, pathogenic Xcc bacteria after 21 d in cold storage post-packinghouse processing. The results from Florida and Argentina demonstrate that common commercial packinghouse
decontamination methods reduce populations of bacteria, and Xcc contamination and survival on fruit. With the bioassays, very low numbers of Xcc bacteria were recovered from apparently healthy fruit in mixtures with symptomatic fruit. The ability to recover viable, pathogenic Xcc bacteria from symptomatic fruit or mixtures containing symptomatic fruit surfaces after packing line disinfection treatment was anticipated. Thus, these treatments were placed in the trial as checks to ensure that Xcc detection methods were adequate.

3.2. Survival of Xcc in fruit wounds

In 2006 populations of bacteria (Xcc) in wounds created by cutting or puncturing the rind of non-processed and processed fruit were tested by bioassay (number of lesions per leaf) in indicator cv. Duncan grapefruit leaves infiltrated with washates from fruit from different sources (A) and time periods in storage (B), and the proportion of infiltration sites on the bioassay plants that developed citrus canker lesions with washates from fruit stored for different periods is shown in (C). The presence of Xcc was tested by bioassay (number of lesions per leaf) in indicator cv. Duncan grapefruit leaves infiltrated with washates from fruit from different sources (A) and time periods in storage (B), and the proportion of infiltration sites on the bioassay plants that developed citrus canker lesions with washates from fruit stored for different periods is shown in (C). The effects of fruit source (D), and time in storage (E) on total bacterial populations of (colony-forming units, cfu ml⁻¹) recovered in the fruit washates were determined by spreading aliquots (100 μl) on KCB agar. The grapefruit were harvested from an orchard in Ft. Pierce, FL and were grouped according to their exposure to Xcc: AHF = apparently healthy fruit from an apparently healthy tree, AHF/IT = apparently healthy fruit from an infected tree, SF = symptomatic fruit, and SF/AHF = symptomatic fruit mixed with apparently healthy fruit from an apparently healthy tree in a 1:4 ratio, respectively. Symptomatic fruit were defined as having 1–5 lesions. Three replicate groups of five fruit from each of the four treatments were assayed to determine the pre-treatment and post-treatment levels of total bacteria and Xcc on fruit. Recovery of total bacteria and surviving Xcc was conducted as described in Fig. 2. Bars with the same letters are not significantly different from each other based on Tukey’s means separation. Data for the main effects of fruit source and storage period are pooled averages of all storage periods or fruit sources, respectively.
grapefruit varied from 5.3 to $6.4 \times 10^4$ cfu ml$^{-1}$ in punctured fruit and from $9.4$ to $9.6 \times 10^4$ cfu ml$^{-1}$ in cut fruit on the day of inoculation (Fig. 7A). After 8 d in cold storage, the populations recovered from the inoculated rind tissue in all treatments decreased ($5.2 \times 10^3$–$1.1 \times 10^4$ cfu ml$^{-1}$) and by 29 d had dropped a further order of magnitude ($3.2$–$9.6 \times 10^2$ cfu ml$^{-1}$). In 2007, an additional treatment of injured fruit left on the tree in the orchard was included. Not all treatments resulted in a consistent decrease in Xcc population over time in 2007. On d 1, bacterial populations for all treatments ranged from 1.0 to $7.7 \times 10^4$ cfu ml$^{-1}$ (Fig. 7B). After 8 d, the populations were lower for all fruit. Thereafter, the populations surviving in the rind of non-processed and processed fruit ranged between $1.8 \times 10^2$ and $2.3 \times 10^3$ cfu ml$^{-1}$. In wounds on fruit kept on the tree, the populations were also variable, ranging from $6.4 \times 10^3$ to $1.3 \times 10^5$ cfu ml$^{-1}$ with time. After 22 d most of the wounded fruit dropped from the tree and could not be assayed. The
data for 2006 were best described using a negative power-law model (Fig. 7C), and all four source groups of fruit showed a similar relationship as recoverable numbers of bacteria declined with time. The data from 2007 were too variable with time to fit a meaningful model describing the changes in the population of \( Xcc \). However, it was clear that minor to severe wounds combined with inoculum on mature fruit surfaces do not develop canker lesions, and \( Xcc \) populations generally decline rapidly, although wounds might occasionally retain more slowly declining \( Xcc \) populations.

3.3. Dispersal of \( Xcc \) from fruit in discarded cull piles

3.3.1. Fort Pierce, Florida

No symptoms developed on any trap plant at any distance from the cull pile of fruit with canker symptoms at any time (Table 1). There were a total of 23 rainfall events during the first experiment in 2006, and 19 rainfall events during the second experiment in 2007. Of these 20 and 16 of these events in 2006 and 2007, respectively, were commensurate with wind speeds in excess of 8 m s\(^{-1}\), providing multiple opportunities for \( Xcc \) dispersal and infection.

3.3.2. Gainesville, Florida

There were 13, 7, 8 and 10 dew or rain events in Experiments 1, 2, 3 and 4, respectively. Mean monthly minimum wind speed ranged from 1.4 to 2.8 m s\(^{-1}\) and mean monthly maximum from 11.2 to 23.3 m s\(^{-1}\). Gusts reached speeds of 35.9 m s\(^{-1}\). No canker symptoms were observed on grapefruit plants from either single lesion source of inoculum from the severely infected fruit from any of the four experiments at any time during the year (Table 1). Even plants with extensive leafminer damage were not infected. No \( Xcc \) was detected on the canker-infected fruit or the leaf surface of the trap plants by plating surface moisture, or bioassay injection–infiltration into grapefruit seedling leaves. In Experiment 1, only one of 12 lesions assayed from the severely infected fruit had viable \( Xcc \) (6.4 \( \times \) 10\(^2\) cfu lesion\(^{-1}\)). No \( Xcc \) was detected in the four lesions from fruit with single lesion. There was no \( Xcc \) detected from fruit in Experiment 2 or 3, however, in Experiment 4, putative \( Xcc \) was detected on two separate occasions in late October on the surface of a single severely infected fruit (1.33 and 7.67 \( \times \) 10\(^1\) cfu ml\(^{-1}\), respectively). Visual observation and confirmation using immunostrips indicated that the bacterial colonies were \( Xcc \); however, injection–infiltration into ‘Duncan’ grapefruit foliage resulted in negative bioassays for \( Xcc \). At the end of Experiment 4, \( Xcc \) was isolated from one fruit with a single lesion (8.0 \( \times \) 10\(^3\) cfu lesion\(^{-1}\)) and confirmed to be pathogenic by the grapefruit injection–infiltration bioassay.

3.3.3. Tucumán, Argentina

Starting in October 2006 through November 2007, there were 10, 12, 16, 18, 15, 18, 6, 9, 3, 2, 1, 2, 11 and 5 d month\(^{-1}\), respectively,
on which rain fell and conditions might have been suitable for Xcc dispersal and infection, with average monthly wind speed ranging from 0.8 to 1.8 m s\(^{-1}\), with occasional gusting exceeding 8 m s\(^{-1}\). There was no infection of any trap plant at any distance from the infected fruit at any time (Table 1). Positive indirect immunofluorescence (IFI) tests were obtained with a total of 10 of the washes at all three distances in February 2007; however, the bioassay did not confirm this. Thus it is likely these were false positives (i.e., not Xcc). No Xcc colonies were identified using the IFI or bioassay tests at any other time.

### 3.3.4. Simulated bacterial dispersal from fruit cull piles and suspended fruit

Only one plant downwind from a cull pile of non-packing line-processed fruit became infected, and developed a single lesion on one leaf (Table 2). This infection event occurred at the highest wind speed (25 m s\(^{-1}\)) and the nearest distance to the infected fruit (0 m), and was associated with leaf injury caused at these extreme wind speeds. No infection developed on plants where the fruit were suspended on strings. Wind-driven simulated rain splash was collected at all distances from the cull piles and suspended fruit (Table 3). No Xcc-positive bacteria were collected in the splash downwind of the cull piles, and only splash from a single treatment replicate resulted in recovery of Xcc in the suspended fruit experiment. This was collected at a wind speed of 25 m s\(^{-1}\) and 2 m downwind from the suspended fruit, and only 3 cfu ml\(^{-1}\) were recovered in this sample (Table 3). Xcc identity was confirmed by a serological positive test using Agdia immunostrips. Furthermore, although the washates of the 10 severely infected fruit produced various morphologically distinct bacterial colonies, none of these tested positive for Xcc using the Agdia immunostrip test.

### 3.4. Natural dispersal from infected citrus peel

A variety and variable number of non-Xcc bacteria were recovered from canker lesions throughout the 36-d period (Table 4).
Table 1
Infection of cv. Duncan grapefruit plants at various distances from a cull pile of grapefruit showing symptoms of citrus canker in multiple geographic locations in 2006 and 2007.

<table>
<thead>
<tr>
<th>Location</th>
<th>Experiment</th>
<th>Distance from inoculum source (m)</th>
<th>No. of plants infected (visual symptoms)</th>
<th>Fruit/leaf wash bioassays</th>
<th>Plating (Fruit/leaf wash)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ft. Piercea</td>
<td>Experiment 1</td>
<td>1</td>
<td>0/8</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>1</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gainesvilleb</td>
<td>Experiment 1</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 2</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 3</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 4</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td>Gainesvilleb</td>
<td>Experiment 1</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 2</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 3</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 4</td>
<td>0.15</td>
<td>0/16</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td>Tucumanc</td>
<td>Oct 2006–Nov 2007</td>
<td>1</td>
<td>0/78</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>0/78</td>
<td>–ve³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>0/78</td>
<td>–ve³</td>
</tr>
</tbody>
</table>

a Experiment 1, as Jun 5–July 21, 2006; Experiment 2, 26 Apr–8 Jun 2007.

b Experiment 1, Jan 5–Mar 28 (plants monitored for disease assessed until Jun 20); Experiment 2, May 1–Jun 21 (plants monitored for disease assessed until Aug 22); Experiment 3, Jul 3–Aug 23 (plants monitored for disease assessed until Oct 5); and Experiment 4; Oct 5–Dec 4 (plants monitored for disease assessed until Jan 25, 2008).

Within each experiment (single lesion vs. multiple lesions) there were four replications. Positive identification of Xcc colonies was made serologically using Agdia immunostrips.

d Each number represents the quantity of Xcc cfu ml⁻¹ collected in each of four replicate runs from these two experiments (B).

Table 2
Combined data for four experiments of the number of canker lesions that developed on cv. Duncan grapefruit trap plants placed downwind from cull piles (A) or suspended groups (B) of severely cankered grapefruit exposed to wind generated using a fan and simulated rain.

<table>
<thead>
<tr>
<th>Wind speed (m s⁻¹)²</th>
<th>Infection (no. of canker lesions/no. of infected leaves/no. of plants)</th>
<th>Distance from cull pile (m)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Cull pile dataa</td>
<td>(total of four runs)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0/0/16</td>
<td>0/0/16</td>
</tr>
<tr>
<td>10</td>
<td>0/0/16</td>
<td>0/0/16</td>
</tr>
<tr>
<td>25</td>
<td>1/1/16</td>
<td>0/0/16</td>
</tr>
<tr>
<td>(B) Suspended fruit data³</td>
<td>(total of four runs)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0/0/16</td>
<td>0/0/16</td>
</tr>
<tr>
<td>10</td>
<td>0/0/16</td>
<td>0/0/16</td>
</tr>
<tr>
<td>25</td>
<td>0/0/16</td>
<td>0/0/16</td>
</tr>
</tbody>
</table>

² Wind was generated using an airboat motor, and rain simulated with garden sprayers.

³ Four plants placed at each distance and for each wind speed tested.

However, no infections developed in grapefruit leaf injection–infiltration bioassays from Xcc-infected peel that were placed in the field for more than 1 d, indicating that although bacteria were detected, they were not Xcc.

3.5. Dispersal from burst infected fruit

No canker symptoms developed on any of the trap plants that were exposed to bits of infected fruits dispersed by violent impact from a baseball bat, despite plants being liberally covered with juice and fruit fragments of symptomatic or apparently healthy fruit (Table 5). When the experiment was repeated, bacteria were isolated (up to 4.14 × 10⁴ cfu ml⁻¹) from leaf washings of individual infected leaves.

| Table 3
The mean total volume of splash collected downwind from cull piles or suspended fruit of severely Xcc-infected grapefruit (A), and the number of Xcc cfu ml⁻¹ collected in each of four replicate runs from these two experiments (B).

<table>
<thead>
<tr>
<th>Wind speed (m s⁻¹)²</th>
<th>Distance from cull pile (m)</th>
<th>(A) Mean total volume of four runs of each experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>794</td>
<td>105</td>
</tr>
<tr>
<td>25</td>
<td>798</td>
<td>291</td>
</tr>
</tbody>
</table>

² Wind was generated using an airboat motor, and rain simulated with garden sprayers and water splash collected in panel traps downwind from the fruit.

²² Each number represents the quantity of Xcc cfu ml⁻¹ collected in each of the four replicate runs of these two experiments (B).
treatment replicates, but none were confirmed to be Xcc. The fruit used for this experiment had lesions that produced viable Xcc, as evidenced by individually tested ground lesions that resulted in recovery of \(0–6.7 \times 10^6\) cfu ml\(^{-1}\).

### 4. Discussion

The bioassay by injection–infiltration of inoculum into attached cv. Duncan grapefruit leaves (Graham and Leite, 2004) used in these studies confirmed isolation of viable, infective Xcc from various groups of fruit, including symptomatic, contaminated and apparently healthy fruit at different times. The total population of bacteria provides additional useful epidemiological information about the distribution and survival of viable, infective Xcc associated with citrus fruit, but must be put in perspective. Throughout the study it became apparent that total bacterial populations recovered from the fruit surfaces did not necessarily correspond to levels of viable, infective Xcc based on the bioassay results. Often numerous, yellow, mucoid bacterial colonies grew on semi-selective KCB media that were visually indistinguishable from Xcc colonies by researchers with many years of experience, but were serologically and pathogenically negative based on monoclonal immunostrip tests and/or a bioassay in susceptible cv. Duncan grapefruit leaves, respectively. The quantity of these (and other) bacteria could exceed that of Xcc, or might occur in the absence of detectable Xcc. In view of this, we relied on the bioassay results using susceptible cv. Duncan grapefruit leaves (Graham and Leite, 2004), and the use of immunostrips for positive identification of viable Xcc isolated in culture, detected in fruit washates, or in wounds. It is important to point out that the results from these various studies in Florida and Argentina corroborate a finding from Japan that citrus fruit are a discontinuity in the pathway, i.e., in the spread of viable, infective Xcc (Shiotani et al., 2009).

#### 4.1. Fruit prewash experiments

Results from the two prewash trials, one with grapefruit in Florida and the other with lemon in Argentina showed that chlorine as a disinfectant alone did not greatly reduce surface bacterial populations. However when chlorine, detergent, or detergent plus chlorine was added as a prewash, followed by a wash usually with SOPP (a disinfectant with detergent activity), there was a reduction in surface bacterial populations. The effect of a prewash was most apparent when SOPP/detergent was included. Prewashing of the fruit, especially with detergent, effectively wets the surface, probably by lowering surface tension, which in turn allows the chlorine greater access to surface Xcc, as well as removing debris such as dirt, sooty mould, and scale insects from the fruit surface that could tie up the chlorine and/or SOPP which potentially reduce the effectiveness of the disinestation treatment. Currently, in citrus packing lines, the normal procedure is to use a prewash of water, or water plus chlorine, followed by a second wash with SOPP. A simple and low-cost recommendation resulting from these studies would be to reverse the procedure and prewash the fruit with detergent (such as SOPP), and follow this by a wash of chlorine with approximately 45 s contact time on the fruit. Based on the results of these experiments this process will more effectively reduce survival of Xcc and other contaminants on fruit after passing through the packing line.

#### 4.2. Packing line experiments

It appears that if fruit are carefully graded to discard symptomatic fruit, the likelihood of asymptomatic fruit either pre-contaminated or becoming contaminated with viable, pathogenic Xcc will be exceedingly low. The reduction in viable Xcc recovery after packing line treatments of grapefruit in Florida and lemon in Argentina was consistent and thus reinforcing. Also, total bacterial population on fruit stored over time with or without (in the case of lemons) processing treatments generally declined. However, on d 21 in the 2007 Florida study, there was an indication that total bacterial populations increased, which might reflect a slow re-colonization of the fruit surface in storage from residual, non-canker and non-pathogenic bacteria that survived the treatment.

### Table 4

<table>
<thead>
<tr>
<th>Variable</th>
<th>Run 1</th>
<th>Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apparently healthy fruit/apparently healthy tree</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic fruit/apparently healthy tree</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic fruit/symptomatic tree</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of bacteria from fruit (cfu ml(^{-1}))</td>
<td>(2.79 \times 10^3)</td>
<td>(0–6.7 \times 10^6)</td>
</tr>
<tr>
<td>Number of bacteria in leaf washates (cfu ml(^{-1}))</td>
<td>(4.14 \times 10^3)</td>
<td>(3.67 \times 10^3)</td>
</tr>
<tr>
<td>Number of infected plants</td>
<td>(0/40)</td>
<td>(0/60)</td>
</tr>
<tr>
<td>Total number of lesions</td>
<td>(0)</td>
<td>(0)</td>
</tr>
</tbody>
</table>

* a Grapefruit fruits were burst using a baseball bat to spatter the fruit parts over the plants.
* b The origin of the fruits was apparently healthy fruit from apparently healthy trees, apparently healthy fruit from symptomatic trees, and symptomatic fruit from symptomatic trees.
* c No samples taken.

### Table 5

The bacterial populations in canker lesions on discarded grapefruit peel under different environmental conditions, and the number of lesions of citrus canker developing on bioassay plants after injection–infiltration of cv. Duncan grapefruit leaves.

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Bacterial population (cfu ml(^{-1}))</th>
<th>Bioassay (lesions leaf(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shadea</td>
<td>Sun</td>
<td>Shade</td>
</tr>
<tr>
<td>1</td>
<td>(3.66 \times 10^7)</td>
<td>(2.01 \times 10^6)</td>
</tr>
<tr>
<td>8</td>
<td>(2.15 \times 10^6)</td>
<td>(1.72 \times 10^6)</td>
</tr>
<tr>
<td>15</td>
<td>(9.84 \times 10^5)</td>
<td>(9.67 \times 10^5)</td>
</tr>
<tr>
<td>22</td>
<td>(6.42 \times 10^5)</td>
<td>(1.14 \times 10^5)</td>
</tr>
<tr>
<td>29</td>
<td>(1.60 \times 10^5)</td>
<td>(1.66 \times 10^5)</td>
</tr>
<tr>
<td>36</td>
<td>(5.92 \times 10^5)</td>
<td>(6.70 \times 10^5)</td>
</tr>
</tbody>
</table>

* a Bacterial leaves injected with aqueous extracts of lesions on discarded grapefruit peels under different environmental conditions.
* b Shade was under a mature grapefruit tree, and the sun location was a 2–3 m adjacent.

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These results are supported by observations on Satsuma mandarin from Japan (Shiotani et al., 2009) where very low levels of Xcc developed, and only a small proportion of lesions produced any inoculum at all, on artificially inoculated, symptomatic and ageing fruit. In that study, externally contaminated, asymptomatic fruit consistently failed to elicit infection of susceptible host material, or to act as a source of detectable Xcc by culturing in vitro.

4.3. Survival of Xcc in fruit wounds

In no cases did harvested mature fruit ever develop lesions after Xcc inoculation. Young canker leaf lesions, the source of inoculum in this study, are known to produce large quantities of Xcc bacteria, and the number of recoverable bacteria that survived within wounds on fruit declined with time for all treatments in 2006; however, in 2007 populations were more variable. Furthermore, injured fruit remaining on the tree tended to sustain higher populations of bacteria longer than harvested fruit. This is likely because of the physiological and biochemical differences in harvested fruit compared to fruit still attached to the tree. Once harvested, fruit begin to senesce, physiological and biochemical changes occur as the fruit breakdown (Bain, 1958; Baldwin, 1993). This breakdown can be slowed to some extent by cold storage and storage under different atmospheric conditions. Both are common postharvest practices with fruit from various tree species but only cold storage is used for citrus. Metabolic changes in harvested fruit compared to fruit still attached to the tree, appear to be detrimental to survival of Xcc. Thus, Xcc populations are not maintained in harvested fruit, but tend to decline to non-detectable levels within a few weeks after harvest. Wounds on harvested fruit containing Xcc inoculum do not lead to canker lesion development, nor do they provide sites for prolonged Xcc survival. Based on these results, the period during which fruit are processed, packed, stored, shipped, and resides in the market place before reaching the consumer (a period of up to several weeks) is likely to be more than sufficient for Xcc populations to decline to non-detectable levels.

4.4. Simulated bacterial dispersal from fruit cull piles and suspended fruit

Although the probability of fruit with undetectable canker lesions moving into the market place is very small, canker-free citrus producing areas consider the risk unacceptable (Shiotani et al., 2009). The concern remains that infected fruit escaping the grading process that are discarded by the consumer, or are disposed of by the importing agent, in the form of ‘cull piles’ might act as a source of inoculum for transmission to canker-free locations. The assumption is that the fruit could be discarded in close proximity to orchards or suburban-grown citrus for this to occur. The risk of these fruit acting as a source of inoculum for infection of the surrounding, healthy trees has not been determined. Culled piles of fruit or discarded fruit do not appear to be significant sources of inocula for infection of susceptible citrus in the field during normal or simulated extreme weather events. Shiotani et al. (2009) also reported that contaminated fruit did not result in infection in the field under natural conditions, nor was viable Xcc detectable on contaminated fruit after brief field exposure to nearby inoculum sources.

Trap plants failed to develop symptoms of canker in all natural weather experiments at all locations, and no viable Xcc was recovered from leaf surface washates. Even in the presence of leafminer damage, which increases susceptibility of the foliage (Christiansen et al., 2007), no canker lesion development was observed on highly susceptible grapefruit trap plants. The simulated extreme weather cull pile experiment was a highly contrived situation designed to provide every possible opportunity for dispersal of Xcc and would be unlikely to occur in most areas, except those locations where hurricanes or tropical storms are common occurrences. The fruit had been individually chosen for severe disease (all fruit had multiple lesions) and it must be stressed they were not processed through the packing line (which appears to reduce lesion activity substantially). At the highest wind speeds a very few bacteria of Xcc were collected in the splash water downwind of the simulated weather event, and only a single lesion developed on one leaf on a plant 0 m from the cull pile at a wind speed of 25 m s⁻¹ in one of four runs of the experiment, and even that lesion was associated with a leaf wound. It is pertinent to note that extreme wind and rain weather conditions potentially conducive to the spread of Xcc occurred in the field experiment in Gainesville, Ft. Pierce and Tucumán, yet no infection was observed at any time on any susceptible trap plant. Thus, infection of susceptible foliage close to a cull pile of fruit with lesions was not observed under field weather conditions, as with a previous study (Shiotani et al., 2009), and the risk of infection by these means, even in simulated extreme weather is exceedingly low. Furthermore, Shiotani et al. (2009) were unable to recover Xcc even after a typhoon blew through an experiment in which contaminated fruit were a source of inoculum in Japan, further demonstrating the unlikelihood of infection arising from this source. Thus, cull piles of diseased, but packing line treated fruit resulted in no infection of surrounding susceptible citrus plants. The fact that current regulation requires presorting of fruit, including removal and disposal of any symptomatic fruit, reduces the risk further of a symptomatic fruit getting to the consumer. However, if that were to happen, the chances of such a fruit being disposed of in a manner that might lead to infection of a susceptible host are unlikely, further reducing the risk.

Lesions on symptomatic fruit often did not produce detectable Xcc, suggesting mature fruit lesions are a poor and insignificant source of Xcc inoculum, and on the rare occasions the lesions are active, they produce fewer bacteria of Xcc than that reported for leaf lesions (Stall et al., 1980; Egel et al., 1991; Timmer et al., 1996; Bock et al., 2005). While canker lesions on young grapefruit may yield significant populations of Xcc (10⁵–10⁶ colony-forming units cm⁻²; Stall et al., 1980), lesions on leaves produce substantially more (10⁴–10⁵ bacteria cm⁻²) depending on the age of the lesion (Egel et al., 1991; Bock et al., 2005). The majority of the research on Xcc bacterial survival and dissemination has been conducted using foliar lesions (Stall et al., 1980; Egel et al., 1991; Bock et al., 2005), and thus does not directly apply to survival in, and dissemination from, fruit lesions.

Foliar lesions initially produce copious quantities of bacteria, but after the first few minutes the quantity drops off and eventually plateaus at a lower level (Timmer et al., 1996; Bock et al., 2005), although foliar lesions have the capacity to repeatedly produce bacteria over many months. In the current study, mature infected, harvested fruit were used as a source of inoculum. The epidemiological significance of harvested fruit lesions as Xcc inoculum sources relates directly to the risk of infection of susceptible host tissue in the vicinity of infected, harvested fruit. In stark contrast to foliar lesions, lesions on harvested fruit often produce low or no Xcc, and when they do, they appear to be less prolific as observed in this study and elsewhere (Shiotani et al., 2009). Coupled with this, cull piles containing cankered fruit, or infected peel cast on the ground, will almost invariably be in an unfavourable location for transmitting Xcc. The physics of the air movement and splash within or close to slower moving or stationary boundary layers near ground level are reasonably well characterized (Aylor, 1974; Aylor et al., 1993). Spray and splash dispersal depends on local conditions, and greater instability results in more dispersal of small droplets (Miller and Stoughton, 2000). Large droplets are most likely
deposited closer to the source, making the transport of splashborne bacteria from ground level a rarer event, even under extreme weather conditions. The results from these natural weather and simulated multi-location experiments suggest that mature citrus fruit with canker lesions are very poor sources of inoculum for susceptible citrus in the field.

Furthermore, the risk of Xcc being dispersed in natural conditions from canker-infected peel discarded in close proximity to healthy, susceptible plants is also very low based on the observation that bioassay test plants did not develop canker lesions with inoculum prepared from naturally-occurring lesions from field-exposed peel beyond the first sampling date. Thus, neither discarded fruit in pulp piles nor discarded fruit peels in the outside environment have demonstrated potential as sources for Xcc dissemination. In both cases, total bacterial and Xcc populations appear to decline rapidly and are not easily disseminated from discarded fruit or peels, confirming other recent observations in Japan (Shiotani et al., 2009).

Similarly, when an infected fruit were struck, burst open, and the fruit pieces and fruit juice dispersed over susceptible plants, no infection developed. A total of 300 fruit were spattered over 300 plants and no canker lesions developed, and no Xcc bacteria were isolated from the surface of these plants. Citrus juice has been shown to be a hostile environment causing a 5-log reduction of bacterial survival (US-FDA, 1998) which may have contributed to lack of Xcc recovery or disease development. Thus, simulation of violently dispersed infected fruit via a baseball bat into susceptible trees was not a pathway for bacterial dissemination and subsequent successful infection of susceptible trees.

4.5. Conclusions and prognosis on fruit as a pathway for the spread of citrus canker

Overall, these evaluations of Xcc bacterial survival and dissemination demonstrate that harvested and packaginghouse-disinfected, citrus fruit with canker lesions are an unlikely pathway through which Xcc inoculum might lead to infection and Xcc establishment in new areas.

References


