Evaluation of asymptomatic citrus fruit (*Citrus spp*.) as a pathway for the introduction of citrus canker disease (*Xanthomonas axonopodis* pv. *citri*)

United States
Department of
Agriculture

Animal and
Plant Health
Inspection
Service

March
2006

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Executive Summary

This evaluation considers the available scientific and other evidence associated with the question of asymptomatic citrus fruit as a pathway for the introduction of citrus canker, a bacterial disease caused by *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin, *et al.*,1995. The evaluation concludes that asymptomatic, commercially produced citrus fruit that has been treated with disinfectant dips and subject to other mitigations is not epidemiologically significant as a pathway for the introduction of citrus canker. Even if infected fruit were to enter a canker-free area with susceptible hosts, the establishment of citrus canker via this pathway is highly unlikely. There is no evidence that asymptomatic fruit is epidemiologically significant as a means for spread. In the unlikely event that viable propagules were present, the environmental and physiological conditions necessary for disease development at the precise time that an infected citrus fruit was placed in close proximity to a susceptible host is highly unlikely. A strong record of empirical data from experience and interceptions further reinforce the conclusion that the likelihood of introducing citrus canker on asymptomatic fruit is extremely low.

Introduction

This document was produced by the Plant Epidemiology and Risk Analysis Laboratory of the Center for Plant Health Science and Technology, USDA Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ). It was completed in response to a request by the Deputy Administrator of PPQ to evaluate scientific and other evidence associated with asymptomatic, commercially produced citrus fruit as a possible pathway for the introduction of citrus canker, a bacterial disease caused by *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin, *et al.*,1995. The study is not limited by the origin of the fruit but rather assumes the fruit may be from any origin where citrus canker occurs; foreign or domestic. Other pests that may be associated with citrus fruit from a particular origin are beyond the scope of this document.

This study focuses on citrus cultivated under specific management practices that include quality control measures implemented to control the incidence of citrus canker in the field to ensure that fruit originates from groves with a low prevalence of citrus canker and that procedures are in place to prevent blemished or symptomatic fruit from being present in shipments of commercial citrus. These requirements include field treatment with copper-based pesticides, subjecting fruit to culling during harvest and packing to remove cankered or otherwise blemished fruit, and treatment after harvest with either a 200 ppm chlorine dip for at least 2 minutes or a 1.86-2.00% sodium orthophenylphenate (SOPP) solution.

APHIS currently prohibits the importation of propagative materials from the family Rutaceae (7CFR § 301.75; 7CFR § 301.83; 7CFR § 319.19; & 7CFR § 319.28). Citrus fruit however is authorized by APHIS for importation from many countries. An import permit is required and the fruit must meet strict requirements for specific pests including fruit flies and diseases such as citrus canker (7CFR §319.28; 7CFR §319.56). Regulations that currently allow the importation of citrus fruit from areas where citrus canker occurs are based on systems approaches.

The Plant Protection Act defines a systems approach as:

"…a defined set of phytosanitary procedures, at least two of which have an independent effect in mitigating pest risk associated with the movement of commodities."[1]

Systems approaches are individualized for each country, commodity, and pest combination. There is no single systems approach for all commodities. Systems approaches may also vary for a particular commodity depending on factors such as the origin, pests of concern, and the availability and feasibility of measures. Systems approaches are typically more complex to design and implement because they require multiple measures. They may also be subject to more frequent review and adjustment based on new information or experience related to one or more aspects of a systems approach.

In the case of citrus fruit from areas where citrus canker occurs, systems approaches authorized by APHIS have included the following main requirements (see also Figure 1):

- Fruit must be grown in a canker-free area surrounded by a non-export buffer zone;
- Surveys of the groves must be conducted on a periodic basis (and prior to harvest) to ensure the area remains canker-free;

[1] The internationally accepted definition of a systems approach is: "The integration of different pest risk management measures, at least two of which act independently, and which cumulatively achieve the appropriate level of phytosanitary protection." (ISPM No. 14; International Plant Protection Convention, 2002)

- Phytosanitary certification for canker freedom and other phytosanitary requirements;
- Freedom of consignments from leaf/stem debris;
- Fruit must be inspected at the packinghouse where it must be exposed to a chemical dip of 200 ppm chlorine (pH 6.0-7.0) for a minimum of 2 minutes; and
- Inspection at the port of entry.

A key aspect of current systems approaches for the importation of citrus fruit is the requirement that production areas are free of the disease and exported fruit is free of symptoms or sign of the disease (asymptomatic). Requiring production area freedom as part of a systems approach may not be technically justified where asymptomatic fruit can be produced if asymptomatic fruit does not provide a pathway for introduction.

Biology and Epidemiology

Citrus canker is caused by the bacteria *Xanthomonas axonopodis* pv. *citri* (Xac). The disease was introduced into the State of Florida in 1912 on infected trifoliate orange seedlings and spread through plant shipments to Alabama, Georgia, Mississippi, Louisiana, South Carolina, and Texas (Civerolo, 1984; Smith et al., 1997; Graham et al., 2004a). The disease was successfully eradicated in Florida by 1933 (Whiteside, 1988) and from all of the U.S. by 1947 (Schubert et al., 2003). Citrus canker reappeared in Florida in 1986, was eradicated again and reappeared in Miami in 1995. Schubert et al. (2003) states "Most scientists believe *X. axonopodis pv. citri* was reintroduced in the early to mid-1980s, but a few speculate that this outbreak might have resulted from perennial holdover from 1910 *X. axonopodis pv. citri* introduction." To date, citrus canker outbreaks in the U.S. have not been associated with imported citrus fruit, although the movement of infested propagative material is recognized as a viable pathway from which introductions have occurred in the past (Shubert et al., 1999).

Citrus canker is a bacterial disease that infects all above ground parts of susceptible hosts, particularly young actively growing leaves, twigs, branches, trunks, thorns, and fruit (Brunings & Gabriel, 2003; Gottwald & Graham, 1992; Graham et al., 1992b; Timmer et al., 2000; Civerolo, 1984) (see also Figure 2). Citrus is the main host of economic importance. Grapefruits (*C. paradisi*), limes (*C. aurantifolia*) and trifoliate orange (*Poncirus trifoliate*) are highly susceptible. Sour oranges (*C. aurantium),* lemons (*C. limon*) and most sweet oranges (*C. sinensis*) are moderately susceptible (Civerolo, 1984; Gottwald et al., 1993; Graham et al., 1992b; Smith et al., 1997; Gottwald et al., 2002a). Most mandarin cultivars (*C. reticulata*) are moderately resistant (Gottwald, et al., 1993; Graham et al., 1992b).

Citrus canker is a disease of tropical and subtropical regions but it can occur and may become established on highly susceptible hosts in temperate and arid regions in the absence of adequate control measures, especially where groves are under frequent irrigation (Civerolo, pers. comm., 2003).

Under optimal conditions citrus canker bacteria multiply 3-4 log units per lesion, and may emerge from the stomata of the infected plant part in as little as five days (Graham et al., 2004b) The highest rate of reproduction occurs on leaves in spring and early summer when conditions are warm (20-30^{o}C) and wet, especially in areas that receive >1000 mm of rain per year (Gottwald et al., 1988; Whiteside, 1988; Muraro, 2001; Smith et al., 1997; Gottwald et al., 2002a; Verniere et al., 2003) . Under conditions of wind-driven rain (greater than 8 m/sec), bacteria are dispersed within trees and from tree to tree (Timmer et al., 2000). The bacteria have been dispersed from 32 meters to a few hundred meters to several miles (Stall et al., 1980; Gottwald et al., 1988, 1992, 2002b; Timmer et al., 2000). In Florida, the pathogen spreads less than 1900 ft

from the source 95% of the time (Gottwald, 2002a; Gottwald et al., 2001; Compton and Fagan, 2000; McElroy, 2000).

Under laboratory conditions, pressurized water soaking of stomata is required for infection to occur, and at least two colony forming units of bacteria per stoma are required to cause lesions on susceptible fruit or leaves (Gottwald & Graham, 1992). The infiltration of bacteria into stomata on fruit or leaves is aided by wind-driven rain in which wind speeds reach in excess of 8 meters per second (Gottwald, et al., 2002a). Thus, high moisture and wind conditions and the presence of sufficient inoculum and susceptible hosts are essential for epidemic development Gottwald & Graham, 1991).

Bacteria can proliferate on leaves of susceptible citrus cultivars (Gottwald & Graham, 1992; Verniere et al., 2003). Lesions generally increase over time ceasing expansion 20-30 days after initial inoculation (Graham et al., 1992 b). Citrus canker is especially devastating in areas with hot, humid climates and frequent wind-driven rain (Civerolo, 1984; Timmer et al., 2000; Schubert et al., 2001). Leaf infection can occur within 14-21 days after shoots begin to develop (Graham et al., 1992a). Unwounded fully expanded leaves are less susceptible to colonization (Graham et al., 1992a; Timmer et al., 2000). Graham et al. (1992) state, "Because leaves are highly susceptible to stomatal infections only from two thirds to full expansion stage, cultivars with a greater frequency, size, and duration of leaf flushes, such as grapefruit, are more "field susceptible" to Asiatic citrus canker than less vigorous citrus cultivars. Hence, epidemics of citrus canker occur when leaf flushes coincide with weather conditions that are ideal for infection and spread of *X. c. citri*." Leaf infection occurs during enlargement (Civerolo, 1984), over a short time interval of susceptibility with the majority of infection occurring within the first 6 weeks (Timmer et al., 2000; Nat. Plant Board, 2002).

Fruit are susceptible to infection from petal fall to 90-120 days thereafter and can be exposed to several infection cycles (Schubert et al., 2001; Civerolo, 1984; Gottwald et al., 2002a; Timmer et al., 2000). Fruit 2-6 cm in diameter remain highly susceptible to infection; however susceptibility reduces as fruit develop (Civerolo, 1984; Graham et al., 1992b; Verniere et al., 2003; Graham et. al., 2004a). Lesions can serve as a source of inoculum on the same fruit (Civerolo, 1894). Mature, unwounded fruit are not known to be susceptible to infection (Civerolo, 1984; Timmer et al., 2000). Mature fruit and leaves are relatively immune to citrus canker because a waxy cuticle builds up on maturing fruit and leaves causing the stomata and other natural openings become plugged and less susceptible to pathogen penetration (Albrigo, 1976; Graham et al., 1992b; Timmer et al., 2000; Nat. Plant Board, 2002). Furthermore, citrus fruit have fewer stomata per area than the same amount of leaf surface (Albrigo, 1976). Due to the lowered susceptibility of mature tissue, young trees generally have more canker than older trees (Whiteside, 1988; Timmer, et al., 2000).

The Asian leafminer (*Phyllocnistis citrella* Stainton) synergistically interacts with canker by providing wounds that serve as infection courts in leaves and, to a lesser extent, fruit (Gottwald, et al., 2002a, b; Schubert et al., 2001). Leafminer-damaged leaves have much more expansive cankers, which contributes to inoculum production and subsequent disease spread (Gottwald, et al., 2002a; Verniere et al., 2003). Verniere et al. (2003) states "The combination of *X. axonopodis* pv. *citri* and the leaf miner can lead to significant field infection even on highly resistant cultuvars and species of citrus such as calamondin and kumquat (T. R. Gottwald, unpublished)" (Nat. Plant Board, 2002).

Bacteria survive in lesions formed on above-ground parts of susceptible hosts including fruit still attached to the tree, leaves, twigs, stems, and the bark of the trunk and possibly for short periods of time (<62 days) on non-citrus weed and grass hosts (Leite and Mohan, 1990). Bacteria produced in leaf and twig lesions are epidemiologically significant for secondary infections (Pruvost et al., 2001) and stem lesions can act as reservoirs of inoculum for longer periods than fruits and leaves (Leite and Mohan, 1990; Verniere et al., 2003). Bacteria may survive a maximum of 120 days on decomposing plant litter (fallen fruit, leaves, and limbs) on the soil surface (Graham et al., 1987; Civerolo, 1984; Leite and Mohan 1990; Gottwald et al., 2002a; Schubert et al., 2001). The bacterium is a facultative saprotroph (Goto et al., 1978; Graham et al., 1987); viable bacteria have been isolated from 5-7 year-old stem lesions (Schubert et al., 2001). Asymptomatic citrus leaves are believed to harbor citrus canker bacteria for several months, and late-season lesions are capable of sustaining bacteria from one season into the next season (Schubert et al., 2001). These conditions allow for the persistence of citrus canker bacteria in areas where the disease is already established and provide inoculum for disease spread (Graham et al., 1987).

Prophylactic sprays of copper oxychloride or other copper-containing compounds provide protection against initial infection in canker-endemic areas during growth flushes and fruit development (2-6 cm diameter) (Stall et al., 1980; Leite and Mohan, 1990; Graham et al., 2004a; Muraro et al., 2001; Das, 2003). Additional copper fungicide sprays are often needed to avoid symptoms in generally infected areas since the first spray usually wears off and may not effectively limit infection potential (Stall et al., 1980; Leite and Mohan, 1990; Gottwald et al. 2002a). Graham and Gottwald, (1991) state "In the absence of windbreaks, copper applied at 1-month intervals slightly reduced canker spread during windblown-rain events, but copper did not affect canker spread and disease severity when used in conjunction with windbreaks (L.W. Timmer and T.R. Gottwald, unpublished)."

Post-harvest treatments (e.g., sodium hypochlorite dips) of artificially inoculated fruit have been shown to eradicate *Xanthomonas campestris* pv *vesicatoria*, a closely related bacteria (Brown & Schubert, 1987). 200 ppm Cl applied for 2 minutes to infested fruit collected from the field showed that natural bacterial populations were lowered by 77-99%. While a relatively high number of natural bacteria and fungi survived, no naturally occurring *Xanthomonas* populations could be detected or isolated after treatment (Stapleton, 1986, Schubert et al. 1999). Similar results were obtained using chlorine and/or sodium orthophenylphenate (SOPP) treatments (Obata, 1969).

Spread Potential

Citrus canker spread from an initial 50 square mile to 1,265 square miles area in the first five years after detection of the epidemic that was centered in the Miami area (Gottwald et al., 2001). This spread was due mainly to conducive environmental conditions that included storms with high winds and wind-driven rain on a regular basis. For example a thunderstorm in 1989 was recorded to have spread inoculum and resulted in the establishment of four new disease foci 230 to 810 m from the infected source trees (Graham et al. 2004b). The disease would be expected to be less likely to develop and easier to eradicate in areas where such conditions are less frequent (Graham 1991). The "Mediterranean" climate (dry summers) of California (except in microclimates with highly susceptible cultivars such as along the coast between San Diego and Ventura; (Civerolo, pers. comm. 2003) and the arid climate of Arizona make establishment of citrus canker less likely, but possible, given that citrus canker is known to occur in the Arab Peninsula on a highly susceptible variety of Mexican lime (Das, 2003; Graham et al., 2004a). The spread of citrus canker in Texas and Louisiana would likely be lower than for Florida because the climate would not support inoculum build-up during summers with much less wind-blown rain (Gottwald, 1988; Smith, et al., 1997; Stall et al., 1980

USDA, 1997) (see also Figure 2). However, the rate of spread would also depend upon major climatic events if the pathogen were to reach Louisiana or Texas. Experience with the spread of canker in Florida clearly indicates that transmission is closely linked to major climatic events such as hurricanes.

Long distance spread of the pathogen is known to occur via the movement of infected planting and propagating material, such as budwood and rootstock seedlings or budded trees from nurseries (Smith et al., 1997; Gottwald et al., 2002a; Graham et al., 2004a; Timmer et al., 2005). There is no confirmed record of seed transmission (Smith et al., 1997). Contaminated personnel, clothing, equipment, tools, field boxes and other items associated with harvesting and post-harvest handling of fruit may also provide a means of long distance dissemination especially when trees are wet (Civerolo, 1984; USDA, 1997; Timmer et al., 2005). Bacteria can survive in plant debris and soil (Goto et al., 1978; Graham et al., 1987), thus the removal of leaves, fruit and limbs from the grove floor and the removal of infected trees help reduce inoculum and diminish spread potential.

The movement of diseased fruit has often been considered to be a potential means of long distance spread of the pathogen, however there is no authenticated record of this being related to the epidemiology of the disease despite a long global history of commercial trade in citrus fruit (Smith et al., 1997). Likewise, infected cull fruit and processed fruit pulp have also been identified as possibly facilitating long-distance spread of the pathogen. The likelihood of this occurring is unknown (Smith et al., 1997). There is no record that either infected fruit with lesions or asymptomatic fruit are epidemiologically significant with respect to the initiation of new infections (Jetter et al., 2000; Canteros, 2004; Argentina, 2005; Risk Assessment, 2005).

Phytosanitary evidence regarding asymptomatic fruit as a pathway

Previous analyses have assessed the likelihood of introduction of the disease from citrus fruit (Schubert, et al., 1999; USDA, 1995; USDA, 1997). Several events have been identified as necessary for the disease to be introduced into a new area on commercial fruit (see Figure 3).
These events include:

(1) Infected/contaminated fruit are harvested;
(2) Inoculum on infected/contaminated fruit survives the packing/treatment process;
(3) Inoculum on infected/contaminated fruit survives shipment;
(4) Infected/contaminated fruit go to a suitable area with conditions for infection;
(5) Inoculum from infected/contaminated fruit encounters a suitable host and is able to incite disease.

Event 1: Infected/contaminated fruit is harvested.

The first event is assumed to be likely if fruit originates in areas where the disease occurs, but the magnitude of the hazard at this stage will depend in large part on the proportion of infected fruit and the nature of the contamination. Groves in infested areas may have citrus canker infected fruit at varying levels depending on the prevalence of inoculum, the susceptibility of the variety, climatic, environmental, and cultural conditions. Presence of the organism on fruit may be associated with lesions, injuries, or blemishes, or it may be epiphytic (contamination). The prevalence of infected or contaminated fruit will depend primarily on the variety, environmental conditions, and field treatment regimes.

Copper protective sprays are a key tool for reducing the incidence of Xac infestation in the field (Stall et al., 1980; Leite and Mohan, 1990; Gottwald et al. 2002a). Well-timed field treatments significantly reduce the incidence of the disease and therefore the level of inoculum and the number of symptomatic fruit in the field. Commercial growers for the fresh fruit market routinely apply copper sprays to control citrus canker (Graham and Gottwald, 1991; Gottwald et al., 2002a; Graham et al., 2004b) and as a general measure to reduce the incidence of other bacteria and fungi that adversely affect the quality and shelf-life of citrus fruit.

Citrus canker is mainly a leaf spotting and fruit blemishing disease. Symptoms of citrus canker on fruit are visually obvious except when uncharacteristic lesions may be associated with injuries or blemishes. Despite decades of commercial and scientific observations, there is no evidence of the existence of undetectable lesions on mature fruit or latency which would result in lesions being expressed after harvest. Mature (not expanding) asymptomatic fruit without injuries or blemishes are not known to express symptoms (Graham, et al., 1992b).

Resistance of fruit to infection by citrus canker is correlated with its stage of maturity. The younger the fruit, the less resistant it is to citrus canker (Graham et al., 1992b). Persistence of the citrus canker pathogen depends on where the canker lesions are located (Schubert et al., 2001). Fruit and leaves that are removed from trees sustain bacterial populations in lesions for 1-2 months in the presence of antagonistic microorganisms (Schubert et al., 2001; Timmer et al., 1996).

Xac is considered a relatively labile bacterium. Epiphytic populations of Xac on citrus tissue drop 3-5 orders of magnitude in 24 hours during experimental testing (Timmer et al., 1996). Timmer et al. also states, “We detected epiphytic [Xac] on asymptomatic plants, but the occurrence of epiphytic populations was not related to subsequent appearance of symptoms”, and additionally, “Our evidence indicates that [Xac] is highly unlikely to persist on hosts or non-hosts in the absence of symptoms for long periods.” Populations decline rapidly even within the lesions of infected fruit after harvest (Civerolo, 1981). Researchers in Argentina sprayed asymptomatic fruit with a bacterial suspension of 10^6 cfu/ml resulting in non-recovery of inoculated bacteria after five days at room temperature under lab conditions (Risk Assessment, 2005). The survival of epiphytic Xac populations on asymptomatic fruit under natural conditions would be expected to be further reduced due to natural environmental conditions being harsher than controlled laboratory conditions.

In commercial operations, diseased, damaged, disfigured, and blemished fruit are culled in both the field and packinghouse. Because the survival of bacteria on non-attached citrus fruit is significantly reduced, the likelihood of spread of citrus canker through bacteria on harvested commercial fruit is considered very low (Graham et al., 1992b). Furthermore, no infection was observed when citrus fruit artificially inoculated with *Xanthomonas axonopodis* pv. *citri* were used as an inoculum source, even under ideal environmental conditions (E. Civerolo, personal communication on unpublished research).

<u>Conclusions for the first event</u>:
The presence of canker infected fruit in an infected orchard is likely; however the prevalence of diseased fruit and of healthy fruit with ephiphytic Xac will be epidemiologically insignificant.

Xac is likely to be present in groves if active infections occur within the export grove or in nearby groves from which the bacteria may be introduced by wind driven rain.

Evidence:

- Under conditions of wind-driven rain (greater than 8 m/sec) bacteria are dispersed within trees and from tree to tree (Timmer et al., 2000).
- The bacteria have been dispersed from 32 meters to a few hundred meters to several miles (Stall et al., 1980; Gottwald et al., 1988, 1992, 2002b; Timmer et al., 2000).
- In Florida, the pathogen spreads less than 1900 ft from the source 95% of the time (Gottwald, 2002a; Gottwald et al., 2001; Compton and Fagan, 2000; McElroy, 2000).

Under the systems approach, the prevalence of citrus canker diseased fruit will be low.

Evidence:

- Copper protective sprays are a key tool for reducing the incidence of Xac infestation in the field (Stall et al., 1980; Leite and Mohan, 1990; Gottwald et al. 2002a).
- Prophylactic sprays of copper oxychloride or other copper-containing compounds provide protection against initial infection in canker-endemic areas during growth flushes and fruit development (2-6 cm diameter) (Stall et al., 1980; Leite and Mohan, 1990; Graham et al., 2004a; Muraro et al., 2001; Das, 2003).

Infected fruit express detectable symptoms and are likely to be culled or not harvested.

Evidence:

- Citrus canker is mainly a leaf spotting and fruit blemishing disease. Symptoms of citrus canker on fruit are visually obvious except when uncharacteristic lesions may be associated with injuries or blemishes (No reference cited in document).
- Mature (not expanding) asymptomatic fruit without injuries or blemishes are not known to express symptoms (Graham, et al., 1992b).
- In commercial operations, diseased, damaged, disfigured, and blemished fruit are culled in both the field and packinghouse (No reference cited in document).

Fruit contaminated by epiphytic populations of Xac is unlikely to have a significant role in disease spread.

Evidence:

- Xac is considered a relatively labile bacterium (No reference cited in document).
- Epiphytic populations of Xac on citrus tissue drop 3-5 orders of magnitude in 24 hours during experimental testing (Timmer et al., 1996). Timmer et al. also states “we detected epiphytic [Xac] on asymptomatic plants, but the occurrence of epiphytic populations was not related to subsequent appearance of symptoms”, and additionally “our evidence indicates that [Xac] is highly unlikely to persist on hosts or non-hosts in the absence of symptoms for long periods.”
- Researchers in Argentina sprayed asymptomatic fruit with a bacterial suspension of 10^6 cfu/ml resulting in non-recovery of inoculated bacteria after five days at room temperature under lab conditions (Risk Assessment, 2005).

<u>Event 2: Inoculum on infected/contaminated fruit survives the packing/treatment process.</u>

The second event is the likelihood of inoculum surviving the packing and treatment process. Harvested fruit is transported to the packing house where it is subjected to one or more visual inspections. Field culling would be expected to have removed nearly all symptomatic and blemished fruit prior to arrival. Visual and/or mechanical inspections remove any remaining symptomatic or blemished fruit as well as misshapen, off-color, damaged and otherwise low quality fruit. The efficacy of this process varies slightly depending

on the facility and system, but APHIS' experience in the United States, Argentina, Japan, and other countries shows that it can be highly effective for removing symptomatic fruit.

Citrus fruit is washed when it enters the packing house as a normal part of any packing process (see also Figure 4). This washing usually includes mechanical brushing and detergent (at the very least) which will reduce epiphytic bacterial populations (Schubert, et al., 1999) by 1 to 2 orders of magnitude. The drying process (with hot air at 58 °C for 2.5 minutes) after washing further reduces populations of epiphytic Xac (Schubert et al., 1999).

The application of a finishing wax further reduces the likelihood that any remaining bacteria will be available to cause new infections (Stapleton, 1986). When the washing process includes a disinfectant such as 200 ppm chlorine or SOPP, Xac populations are reduced to undetectable levels (Brown and Shubert, 1987; Graham and Gottwald, 1991; Obata, 1969). The post harvest treatments such as chlorine and SOPP guarantee the complete eradication of epiphytic Xac on fruits without symptoms (Canteros, 2004). Additionally, their joint use (common practice in export pack houses) substantially reduces the Xac viability in lesions. (Brown and Schubert, 1987; Canteros, Naranjo and Rybak, 2000; Argentina, 2005).

The use of 200 ppm Cl for 2 minutes reduces natural bacterial populations on citrus fruits from 77-99%, and natural Xac populations were not detected at all on disinfected fruit (Stapleton, 1986). The estimated reductions in bacterial populations on the surface of citrus fruits that have been brushed, washed, and disinfected with Cl or SOPP would be between 3 and 7 orders of magnitude, according to the research cited above. According to research by Verdier et al. (Risk Assessment, 2005), bacterial populations on artificially infested fruits were $1.7x10^3$ cfu/ml and 39 cfu/ml on naturally infested asymptomatic fruit prior to post harvest treatment in Uruguay. After treatment these levels were reduced to 8 cfu/ml for artificially infested fruit and 0.06 cfu/ml respectively for naturally infested fruit. Bacterial populations used for artificial inoculation of fruit are at extremely high levels never found in natural conditions (Risk Assessment, 2005).

<u>Conclusions for the second event:</u>
The likelihood of inoculum on infected fruit surviving the packing and treatment process is very low.

Symptomatic fruit (the main source of inoculum) is highly unlikely to pass through the packing process.

Evidence:
- Field culling would be expected to have removed a high percentage of symptomatic and blemished fruit prior to arrival.
- Visual and/or mechanical inspections remove virtually all remaining symptomatic or blemished fruit as well as misshapen, off-color, damaged and otherwise low quality fruit.

Standard packinghouse procedures and postharvest treatments prescribed by the systems approach will remove, devitalize and/or immobilize the pathogen.

Evidence:
- Washing and brushing will reduce epiphytic bacterial populations (Schubert, et al., 1999) and the drying process (with hot air at 58 °C for 2.5 minutes) after washing further reduces populations of epiphytic Xac (Schubert et al., 1999).
- Washing with a disinfectant such as 200 ppm chlorine or SOPP reduces Xac populations to undetectable levels (Brown and Shubert, 1987; Graham and Gottwald, 1991; Obata, 1969).

- Post harvest treatments such as chlorine and SOPP guarantee the complete eradication of epiphytic Xac on fruits without symptoms (Canteros, 2004).
- Joint use of chlorine and SOPP substantially reduces the Xac viability in lesions. (Brown and Schubert, 1987; Canteros, Naranjo and Rybak, 2000; Argentina, 2005).
- The use of 200 ppm Cl for 2 minutes reduces natural bacterial populations on citrus fruits from 77-99%, and natural Xac populations were not detected at all on disinfected fruit (Stapleton, 1986).
- The application of a finishing wax further reduces the likelihood that any remaining bacteria will be available to cause new infections (Stapleton, 1986).

Event 3: Inoculum on infected/contaminated fruit survives shipment.

The third event is concerned with the survival of inoculum on fruit during shipping. Bacteria that may survive the packing process have not been shown to occur in the surface of citrus fruit or under the wax coating applied to the fruit. Any surviving bacteria are very unlikely to be capable of causing disease development under these circumstances. Fruit-to-fruit post-harvest spread of bacteria has never been documented. According to E. Civerolo, (1997) it has not been shown that Xac latent populations produce symptoms in harvested fruit (Risk Assessment, 2005), and there is no evidence of an epiphytic growth stage for citrus canker (Brunings & Gabriel 2003).

Xac bacteria associated with lesions may continue to multiply as long as lesions continue to expand (Graham et al. 1992a; Graham et al. 2004b). Bacteria survive only in the margin of the lesions in citrus leaves and fruit until it falls off or is removed from the tree (Graham et al. 2004b), thus Xac bacteria do not increase in number on fruit once the fruit is removed from the tree.

Conclusions for the third event:
If bacteria survive the packing process, they will have a high rate of mortality during shipping.

Evidence:

- Xac is considered a relatively labile bacterium (No citation given in document).
- Epiphytic populations of Xac on citrus tissue drop 3-5 orders of magnitude in 24 hours during experimental testing (Timmer et al., 1996). Timmer et al. also states “we detected epiphytic [Xac] on asymptomatic plants, but the occurrence of epiphytic populations was not related to subsequent appearance of symptoms”, and additionally “our evidence indicates that [Xac] is highly unlikely to persist on hosts or non-hosts in the absence of symptoms for long periods.”
- Populations decline rapidly even within the lesions of infected fruit after harvest (Civerolo, 1981).
- Researchers in Argentina sprayed asymptomatic fruit with a bacterial suspension of 10^6 cfu/ml resulting in non-recovery of inoculated bacteria after five days at room temperature under lab conditions (Risk Assessment, 2005). The survival of epiphytic Xac populations on asymptomatic fruit under natural conditions would be expected to be further reduced due to harsher environmental conditions.

Bacteria that survive on or in fruit after post harvest treatment will not multiply or cause disease development in the treated fruit.

Evidence:

- There is no evidence of an epiphytic growth stage for citrus canker (Brunings & Gabriel 2003).
- Fruit-to-fruit post-harvest spread of bacteria has never been documented (No citation).
- There is no authenticated record for diseased fruit playing a role in the epidemiology of citrus canker disease (Smith, et al., 1997).

- There is no record that either infected fruit with lesions or asymptomatic fruit are epidemiologically significant with respect to the initiation of new infections (Jetter et al., 2000; Canteros, 2004; Argentina, 2005; Risk Assessment, 2005).

Event 4: Infected/contaminated fruit go to a suitable area with conditions for infection.

The fourth event involves shipment of Xac-infected fruit to a habitat suitable for disease development. Fruit that arrives in the United States does not normally arrive at a single port; it is instead distributed according to market demand. Demographics derived from United States Census data may be useful in predicting the distribution of imported citrus fruit by indicating population centers where demand may be greatest. Three of the four most populous States in the United States, Florida, Texas, and California, are in the southern tier of States where the climate most closely resembles the native climates for the pests analyzed (U.S. Census, 2000). These three States account for approximately 25 percent of the total U.S. population (U.S. Census, 2000). If we assume that citrus is distributed proportionally across the United States according to population, then it is reasonable to assume that some fruit will be shipped to these States. However, only a small portion of the area of these States is actual citrus producing areas and an even smaller portion of the area has a climate suitable for canker disease development. Similar analyses apply to other countries where citrus may be grown.

Conclusions for the fourth event:
Although shipment of imported infected fruit to a suitable habitat is possible, the fraction that would be shipped to suitable habitat is small.

Evidence:

- Only a relatively small proportion of the citrus growing areas in the United States are at risk.
- Citrus canker is a disease of tropical and subtropical regions but it can occur and may become established on highly susceptible hosts in temperate and arid regions in the absence of adequate control measures, especially where groves are under frequent irrigation (Civerolo, pers. comm., 2003).
- The disease would be expected to be less likely to develop and easier to eradicate in areas where warm rainy conditions are less frequent (Graham 1991).
- The "Mediterranean" climate (dry summers) of California (except in microclimates with highly susceptible cultivars such as along the coast between San Diego and Ventura; (Civerolo, pers. comm.) and the arid climate of Arizona make establishment of citrus canker less likely, but possible.

Event 5: Inoculum from infected/contaminated fruit encounters a suitable host and is able to incite disease.
The fifth event is that the Xac inoculum on imported fruit encounters a suitable host within the suitable habitat. Furthermore, the host must be at a stage that is susceptible to canker, with proper environmental conditions for disease to occur. Transmission scenarios include:

1. A host plant caretaker eats a canker-infected fruit and trims citrus trees soon thereafter. This mode of transmission is unlikely because the acidic citrus juice is bactericidal (Schubert et al. 1999).

2. The peel of a canker-infected citrus fruit is left near a host plant, in a compost pile, or in a trash can in close proximity to a susceptible host. There is a very small chance of successful transmission in this scenario (Schubert et al. 1999). However, rapid decomposition dramatically reduces the chance of transmission over time (Goto, et al., 1978). Furthermore, for bacteria to move from the ground, a compost pile, or a trash can near susceptible tissue on branches, fruit or leaves would

require rain and winds in excess of 18 MPH (8 meters/second) as is needed in tree to tree transport (Timmer et al., 2000).

The development of disease requires the inoculum to successfully infect susceptible hosts. Assuming the inoculum is available, the following must also occur for disease development to occur:

- A susceptible host must be available;
- The host must be exposed during a time when infection can occur (varies with cultivar);
- Free water must also be available on the surface of the citrus plants from rain; and
- Wind speed must reach 8 m/s.

Conclusions for fifth event:
It is unlikely that viable bacteria from an infected fruit would encounter a suitable host under the conditions required for disease development.

Evidence:

- Known hosts of Xac are almost exclusively in the Rutaceae particularly *Citrus* (Smith, et al., 1997).
- Leaf infection occurs during enlargement (Civerolo, 1984), over a short time interval of susceptibility with the majority of infection occurring within the first 6 weeks (Timmer et al., 2000).
- Fruit are susceptible to infection from petal fall to 90-120 days thereafter and can be exposed to several infection cycles (Schubert et al., 2001; Civerolo, 1984; Gottwald et al., 2002a; Timmer et al., 2000). Fruit 2-6 cm in diameter remain highly susceptible to infection, however susceptibility reduces as fruit develop (Civerolo, 1984; Graham et al., 1992b; Verniere et al., 2003; Graham et. al., 2004a).
- Mature, unwounded fruit are not known to be susceptible to infection (Civerolo, 1984; Timmer et al., 2000).
- Mature fruit and leaves are relatively immune to citrus canker because a waxy cuticle builds up on maturing fruit and leaves causing the stomata and other natural openings become plugged and less susceptible to pathogen penetration (Albrigo, 1976; Graham et al., 1992b; Timmer et al., 2000; Nat. Plant Board, 2002).
- The infiltration of bacteria into stomata on fruit or leaves is aided by wind-driven rain in which wind speeds reach in excess of 8 meters per second (Gottwald, et al., 2002a). Thus, infection requires high moisture and wind conditions and the presence of sufficient inoculum and susceptible hosts.
- Fruit-to-fruit post-harvest spread of bacteria has never been documented (No citation).
- There is no authenticated record for diseased fruit playing a role in the epidemiology of citrus canker disease (Smith, et al., 1997).
- There is no record that either infected fruit with lesions or asymptomatic fruit are epidemiologically significant with respect to the initiation of new infections (Jetter et al., 2000; Canteros, 2004; Argentina, 2005; Risk Assessment, 2005).

Other analyses have speculated on the amount of prohibited plant materials that are not intercepted upon entry (USDA-APHIS-PPQ-AQIM databases, Meissner, et al., 2003). The total number of citrus canker infested fruit that enters the U.S. undetected or not inspected is unknown, but numerous years of interception data show that citrus canker (*Xanthomonas axonopodis* pv *citri)* has been intercepted more than 1,900 times, while the genus *Xanthomonas* has been intercepted over 13,000 times (APHIS PIN309) at U.S. ports of entry, mainly associated with passenger baggage.

Based on these studies and the constraints of port inspectors to inspect baggage, the entry of citrus canker-infected material thousands of times in the past is conceivable. No canker outbreaks have ever been associated with such entry however. A large portion of the fruit moving illegally probably has not been subject to a "systems approach" (citrus in baggage is frequently from dooryards), but no evidence has been found that mature citrus fruit act as a pathway for the transmission of the disease and such movement has not been associated with an outbreak of the disease anywhere in the world.

Empirical data from a significant history of programmatic experience and interceptions demonstrates that even with a high frequency of unauthorized citrus fruit imports, outbreaks linked to fruit have never been observed. Imports that are authorized based on a systems approach have demonstrated that operational parameters for mitigating risk are feasible and highly effective in excluding blemished or symptomatic fruit. Citrus canker infected material can possibly enter the United States on citrus propagative material (Smith et al., 1997). However, epiphytic Xac on citrus leaves is also not an important source of inoculum (Timmer et al., 1996).

Summary

A systems approach that excludes fruit with blemishes or signs of citrus canker and uses an effective surface disinfection treatment has been demonstrated to be a highly effective means to mitigate the likelihood of fruit as a pathway for the introduction and spread of citrus canker. Key aspects of this mitigation strategy are:

- Field management (primarily copper sprays) to control the incidence of the disease in the field;
- Culling of symptomatic and blemished fruit in the field and packinghouse; and
- Treatment with chlorine or SOPP wash.

The wash treatment eliminates epiphytic contamination as a hazard provided the treatment is done correctly. The remaining hazard is the small possibility that symptomatic fruit escapes culling. In this case, the fruit would be expected to have a very low inoculum level as a result of being harvested, washed, treated, and waxed. Any inoculum remaining or produced afterward would rapidly lose viability. In the event that a symptomatic fruit escapes with viable inoculum, a series of events must occur for citrus canker to be introduced into a new area. The Xac-infected fruit must also arrive in an area where susceptible hosts occur and enter a suitable habitat at the right time and under the right environmental and cultural conditions for disease development. It is extremely unlikely that fruit will encounter this specific range of conditions necessary to infect another plant.

<u>These points consistently argue that asymptomatic fruit is not epidemiologically significant as a pathway for introducing citrus canker if produced under the conditions of a systems approach consistent with the assumptions of this analysis.</u>

Uncertainty

The possibility that asymptomatic fruit can be a pathway for introduction under natural conditions has never been authenticated despite repeated experiments to this effect (Civerolo, pers. comm. 2005). There is no scientific evidence that an outbreak can originate from asymptomatic commercial fruit (Civerolo, 1997). Experiments suggest that even when conditions are suitable, the likelihood of transmission of citrus canker from *X. axonopodis* pv. *citri* in incipient citrus canker lesions or from epiphytic *X. axonopodis* pv. *citri* on citrus fruit is low (E. Civerolo, personal communication on unpublished research).

Further research could strengthen the evidence surrounding the epidemiological significance (or lack there of) of *Xanthomonas axonopodis pv. citri* (Xac) on symptomatic and asymptomatic commercial citrus fruit. Despite the presence of citrus canker in the United States and elsewhere for many years, research gaps exist. The following identifies key research needs that would strengthen regulatory decision making.

Issue: Can symptomatic fruit that has been treated (with SOPP, chlorine, or other appropriate disinfectant) transmit the bacteria that cause the disease, (i.e., can disease be incited on healthy trees or seedlings from infected, symptomatic fruit that has been treated post-harvest)?

Reason: Phytosanitary requirements are not justified if treated fruit with lesions are not a pathway.

Issue: What are the differences in the efficacy of various post-harvest treatments (e.g., SOPP, chlorine, etc) at rendering symptomatic fruit epidemiologically insignificant?

Reason: Chlorine is a common standard; it is also highly unstable. Alternative products and their activity need to be better understood.

Issue: What is the relationship between the proportion of symptomatic fruit in the field and the proportion of infected fruit after post-harvest culling?

Reason: Infected groves or partially infected groves may be part of an exporting area. Evidence shows that in Argentina, up to 20% of the fruit for export is harvested with canker blemishes. It is therefore useful to understand how effective post-harvest culling is in producing asymptomatic fruit. If, for example, culling is highly effective regardless of how "dirty" the incoming fruit is, then field practices are less important in a systems approach.

Issue: What is the efficacy of specific packinghouse equipment and procedures in removing blemished fruit?

Reason: Optical scanners in commercial use have been reported to accurately detect less than 1 in 100,000 *blemished* fruit (only a small proportion of the blemishes might be attributed to citrus canker). This would appear to be a highly effective mitigation. However, data have not been provided on the consistency, effectiveness, or other characteristics of optical scanning systems, or their relative effectiveness compared to manual systems.

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Figure 1. Systems approach applied to orange production (Illustrations: H. Hartzog)

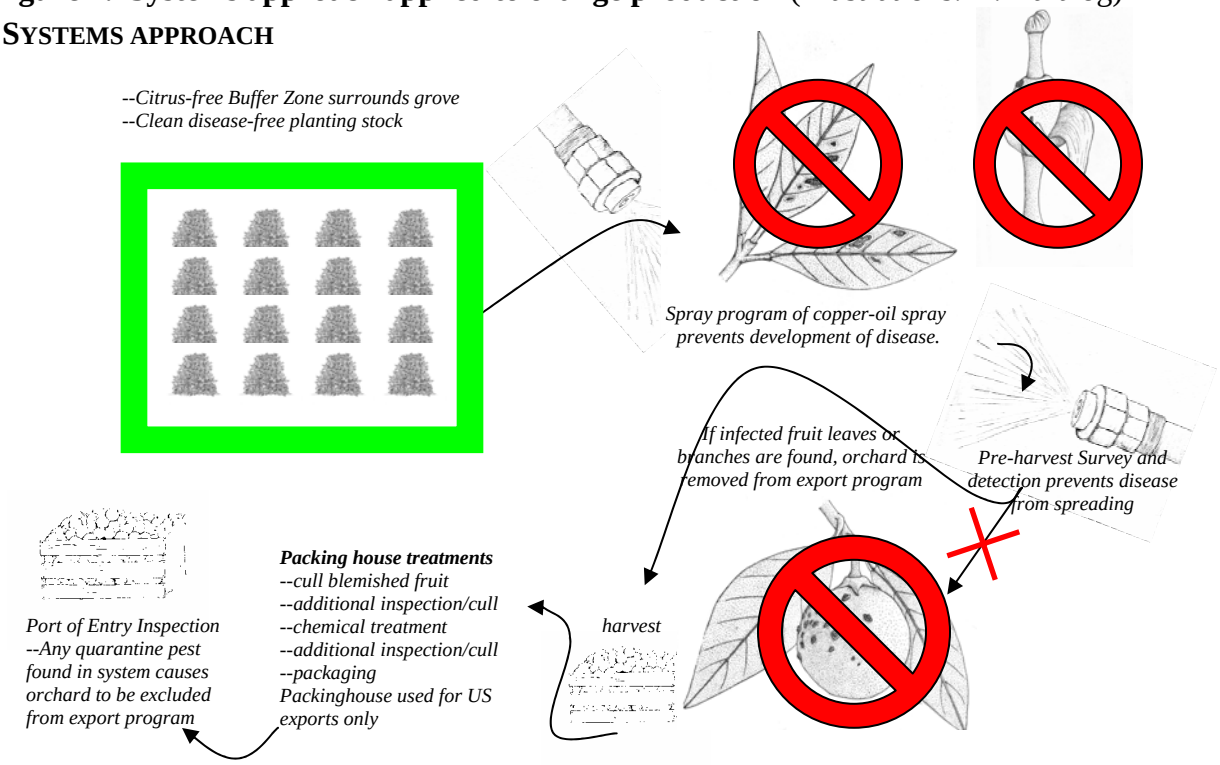

Figure 2. Life cycle of citrus canker (*Xanthomonas axonopodis* pv. c*itri*)

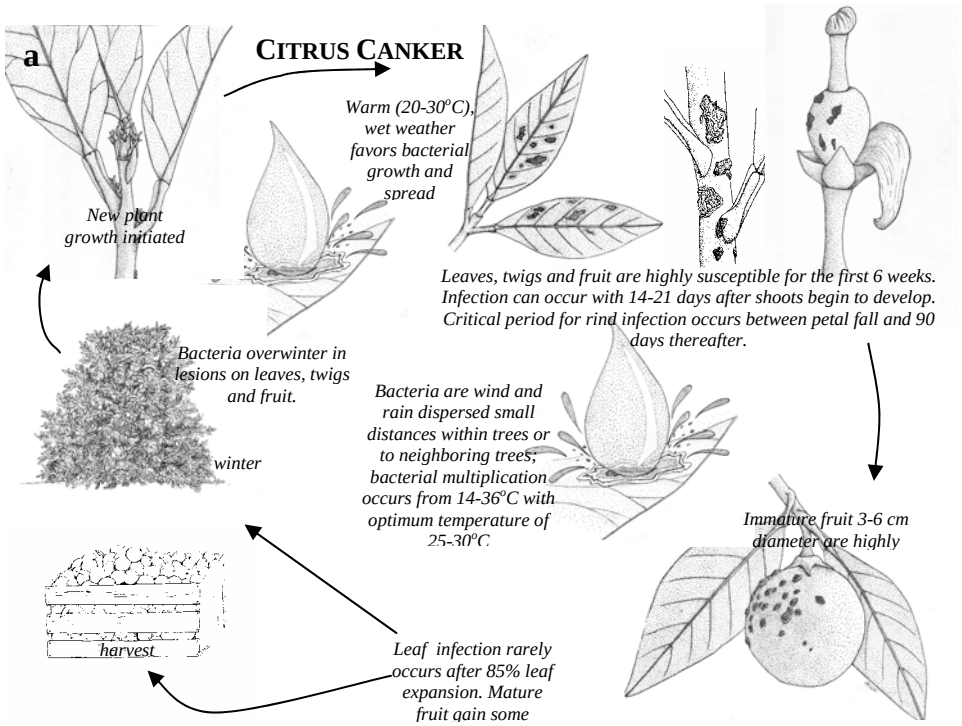

Figure 3. Events leading to introduction

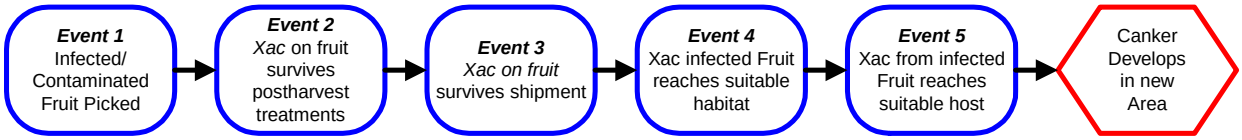

Figure 4. Packinghouse procedures