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

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Executive Summary

This evaluation considers available scientific, expert, and empirical evidence associated with the question of whether asymptomatic citrus fruit is a pathway for the introduction of citrus canker, a bacterial disease of citrus caused by *Xanthomonas axonopodis* pv. *citri* (Hasse) (Vauterin *et al.*, 1995). The evaluation concludes that asymptomatic, commercially produced citrus fruit, treated with disinfectant dips, and subject to other mitigations, is not epidemiologically significant as a pathway for the introduction of citrus canker.

In the event that infected fruit enter a canker-free area with susceptible hosts, the establishment of the disease via this pathway would require particular environmental and physiological conditions at the precise time that an infected citrus fruit was placed in close proximity to a susceptible host. The combination of conditions necessary for introduction are so difficult to achieve that the likelihood of such occurrence is greater than the baseline exposure represented by unregulated pathways. The conclusions of the evaluation are reinforced by a strong record of empirical data from experience and interceptions.

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 is a revision of a previous document, dated March 2006, completed in response to a request by the Deputy Administrator of PPQ to evaluate evidence associated with asymptomatic[1], commercially produced citrus fruit as a possible pathway for the introduction of citrus canker, a bacterial disease caused by *Xanthomonas axonopodis* pv. *citri* (Hasse). This revision accounts for new information and comments received by APHIS from expert peer review and public comment, which is the result of publishing the original document in the Federal Register with a Notice of Availability from April 6, 2006 to June 5, 2006. The public comment period was subsequently extended by APHIS to July 5, 2006.

This study assumes that commercially produced citrus is cultivated under specific pest management practices wherever citrus canker is present. These pest management practices include field treatments with copper-based pesticides for controlling the incidence of citrus canker. Grove sanitation is another practice used to reduce disease prevalence. It is further assumed that fruit culling procedures during harvest and packing will remove symptomatic, injured, or blemished fruit from commercial shipments of citrus. These measures are combined with a post-harvest surface disinfectant dip treatment of 200 ppm chlorine for at least two minutes or 1.86 - 2.00% sodium orthophenylphenate (SOPP), or both.

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 necessary, 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). A systems approach[2] provides the basis for current regulations that allow the importation of citrus fruit from citrus canker areas.

The Plant Protection Act of 2000 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." Systems approaches are individualized for each country, commodity, and pest combination. There is no single systems approach for all commodities. Systems approaches may vary for a particular commodity depending on factors, such as origin, pests of concern, and the availability and feasibility of measures. Systems approaches are often complex to design and implement because they involve multiple measures; they may also be subject to more frequent review and adjustment due to new information or experience related to one or more aspects of a systems approach.

[1] The term 'asymptomatic' refers to the lack of visible signs or symptoms of the disease.
[2] 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" CPHST Science Panel on Armored Scales (ISPM No. 14; International Plant Protection Convention, 2002).

In the case of citrus fruit from areas where citrus canker disease occurs, systems approaches authorized by APHIS have included the following main requirements:

- 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;
- Phytosanitary certification for canker freedom and other phytosanitary requirements
- Freedom of consignments from leaf and stem debris;
- Fruit must be inspected at the packinghouse, where it is exposed to a chemical dip of 200 ppm chlorine (pH 6.0-7.0) for a minimum of two minutes or a 1.86 – 2.00% sodium orthophenylphenate (SOPP) solution; and
- Inspection at the port-of-entry.

A key aspect of current systems approaches for the importation of citrus fruit is that surveys are performed in production areas to ensure that the disease is below detectable levels. If sufficient evidence exists to conclude that asymptomatic fruit is not a pathway for the introduction of the disease, then the regulatory focus shifts from this requirement for production area freedom to ensuring that only asymptomatic fruit is shipped.

Biology and Epidemiology

This section reviews relevant scientific and empirical evidence related to the biology of the bacteria *Xanthomonas axonopodis* pv. *citri* (Xac), the organism causing citrus canker disease.

The pathogen was introduced into Florida in 1912 via infected trifoliate orange seedlings; it was subsequently detected in Alabama, Georgia, Mississippi, Louisiana, South Carolina, and Texas (Civerolo, 1984; Smith *et al.*, 1997; Graham *et al.*, 2004b). The disease was successfully eradicated in Florida by 1933 (Whiteside, 1988) and nationwide by 1947 (Schubert and Sun, 2003). Citrus canker reappeared in Florida in 1986, was eradicated again, and reappeared in Miami in 1995. Schubert and Sun (2003) state "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 the1910 *X. axonopodis* pv. *citri* introduction." Citrus canker outbreaks in the United States have not been associated with imported citrus fruit; however, the movement of infested propagative material is clearly recognized as a viable pathway from which introductions have occurred in the past (Shubert *et al.*, 1999).

Xac 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) (Figure 1). Citrus is the main host of economic importance. Grapefruits (*Citrus 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), and 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 establish on highly susceptible hosts in temperate and arid regions in the absence of adequate control measures (particularly in groves under frequent irrigation) (Civerolo, pers. comm., 2003).

Under optimal conditions, citrus canker bacteria multiply rapidly ("3-4 log units per lesion"), and may emerge from the stomata of the infected plant part in as few 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°C) and wet, *i.e.*, 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, bacteria dispersal can occur within trees, and from tree to tree (Timmer *et al.*, 2000; Bock *et al.,* 2005). Spread of the pathogen has been recorded from 32 meters to several miles (Stall *et al.*, 1980; Gottwald *et al.*, 1988, 2002b; Timmer *et al.*, 2000). In Florida, pathogen spread was detected within 579 m (1900 ft) from the source 95% of the time (Gottwald, 2002a; Gottwald *et al.*, 2001; Compton and Fagan, 2000; McElroy, 2000).

The bacteria enters young tissue through stomata, natural openings, and wounds[3] (caused by a variety of events). Xac only enters mature tissue through wounds (Goto, 1962). Unwounded leaves and fruit become highly susceptible to the infiltration of bacteria through the stomata when the tissue becomes water congested (Gottwald and Graham, 1992). The infiltration of bacteria into stomata on unwounded fruit or leaves is aided by wind-driven rain, in which wind speeds are in excess of eight meters/second; as a result, this creates enough impact force to drive incoulum infested water into the stomata creating water congestion (Gottwald *et al.*, 2002a; Graham *et al.,* 2004b). In laboratory studies where bacteria penetrate the stomata of water congested tissue, lesions[4] on susceptible fruit or leaves can result from a minimum of at least two colony forming units of bacteria per stoma (an occasional 1 to 1 ratio (Gottwald and Graham, 1992)). Epidemic development occurs on unwounded hosts when leaf flushes coincide with ideal weather conditions (high moisture and wind conditions in excess of eight meters per second), sufficient inoculum, and susceptible hosts (Graham *et al.,* 1992a).

Bacteria can proliferate in lesions 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.*, 1992b). Citrus canker is 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 develop (Graham *et al.*, 1992a). Unwounded, fully expanded leaves are less susceptible to colonization (Graham *et al.*, 1992a; Timmer *et al.*, 2000). As leaves age the pressure required to create water congestion and bacteria penetration increases (Gottwald and Graham, 1992; Gottwald *et al.,* 1993; Graham *et al.,* 1992a). Graham *et al.* (1992a) states: "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

[3] The term "wound" in this document is meant to describe any injury to any external surface of the plant by its being torn, pierced, cut, or broken. Unlike a lesion, the occurrence of a wound does not imply that disease has developed.
[4] The term "lesion" throughout this document is meant to indicate "a localized area of discolored and diseased tissue" following the definition provided by Agrios (1988).

flushes coincide with weather conditions that are ideal for infection and spread of *X. c.* pv. *citri*." Leaf infection occurs during enlargement (Civerolo, 1984), over a short time interval of susceptibility; the majority of infection occurs within the first six weeks of infection (Timmer *et al.*, 2000; Nat. Plant Board, 2002).

Fruits 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; Stall, *et al*., 1981; Goto, 1992; Koizumi, 1981). Fruits 2-6 cm in diameter remain highly susceptible to infection; however, susceptibility is reduced as fruits develop (Civerolo, 1984; Graham *et al.*, 1992b; Verniere *et al.*, 2003; Graham and Leite, 2004a). Lesions can serve as a source of inoculum on the same fruit (Civerolo, 1984). Mature fruit and leaves are relatively resistant to citrus canker because a waxy cuticle builds up on maturing fruit and leaves causing the stomata and other natural openings to become plugged (Albrigo, 1972; Albrigo, 1976; Graham *et al.*, 1992b; Timmer *et al.*, 2000; Nat. Plant Board, 2002) and less susceptible to water congestion and subsequent pathogen penetration. Furthermore, citrus fruit have fewer stomata per area than the same amount of leaf surface (Albrigo, 1976). Verniere *et al.* (2003) reported a disease incidence of zero when inoculating mature fruit either by pin prick or spray inoculation. 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) 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 wounds create suitable microclimates for bacteria and favor Xac development (Chagas *et al*., 2001). Leafminer-damaged leaves have much more expansive cankers, which contributes to inoculum production and subsequent pathogen spread (Gottwald *et al.*, 2002a; Verniere *et al.*, 2003; Goto 1992). Verniere *et al.* (2003) states that "the combination of *X. axonopodis* pv. *citri* and the leafminer can lead to significant field infection even on highly resistant cultivars and species of citrus such as, calamondin and kumquat." Leafminers themselves, however, are inefficient vectors of Xac; the increase of disease incidence is not a result of adult female transmission, but rather the susceptible microclimates created by the larvae (Belasque *et al*., 2005).

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 (Leite and Mohan, 1990). Xac can survive on non-citrus weed and grass hosts, although the degree of epidemiological significance posed by this source is unclear (Goto, 1970; Goto *et al*., 1975a, b; Goto *et al*., 1978; Pereira, 1976; Pereira *et al*., 1978; Stall *et al*., 1980; Serizawa, 1981). Bacteria produced in leaf and twig lesions are epidemiologically significant[5] for secondary infections (Pruvost *et al.*, 2002); and stem lesions can act as reservoirs of inoculum for longer periods than fruits and leaves (Leite and Mohan, 1990; Verniere *et al.*, 2003). The amount of Xac bacteria present within active lesions decreases as the lesions age. Timmer *et al.* (1991) demonstrated in laboratory studies that young lesions (4-6 weeks old) exude ~10^4-10^6 cfu/ml in the first 48 hours of wetting, while older lesions (4-6 months old) exude ~10^2-10^3 cfu/ml in the same time period. Bacteria may survive for a few weeks to several months on decomposing plant litter (fallen fruit, leaves, and limbs) on the soil surface (Graham *et al.*, 1987; Civerolo, 1984; Leite and Mohan,

[5] The term "epidemiologically significant" refers to minimum conditions required for disease transmission.

1990; Gottwald *et al.*, 2002a; Schubert *et al.*, 2001), or in plant material buried in the soil (Graham *et al*., 1987). Survival in decomposing leaves, both in and on the soil surface, is dependent on moisture and temperature (Graham *et al*., 1987; Goto, 1992). Xac bacteria do not survive for long periods of time in the soil in the absence of host material (Graham *et al*., 1989; Leite and Mohan, 1984). The bacterium is a facultative saprotroph on some substrates including citrus debris, *Zoysia* sp. rhizomes, and rice straw (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. Late-season lesions are capable of sustaining bacteria from one season into the next season (Schubert *et al.*, 2001), and bacteria residing on plant material in the soil (as long as 2-4 years) has been shown to infect healthy trees (Pereira *et al*., 1976; Goto *et al*., 1975; Goto, 1992). The aforementioned conditions allow for the persistence of citrus canker bacteria in areas where the disease is already established, providing inoculum for the spread of the pathogen (Graham *et al.*, 1987).

Xac "can survive for many years in infected tissues that have been kept dry and free of soil (Goto, 1992)." If there is not an active disease, i.e. bacteria developing in an active lesion, then the bacteria are short lived. This is due to the destruction of extracellular polysaccharides (EPS). During the infection process, Xac, as reported by Goto (1992): "… produces abundant EPS, either in culture media or in host tissues. The bacterial cells in canker lesions are embedded in a dense matrix of EPS and are dispersed, together with EPS, by rain splash. However, the cells of *X. campestris* pv. *citri* are very unstable when highly diluted in water (i.e., they are rapidly killed when suspended at concentrations lower than $l0^6$ cells/ml). This trait is also found in most xanthomonads, as well as some bacteria of other genera. I call this phenomenon lethal dilution effect.' The lethal dilution effect is overcome by addition of EPS at a concentration as low as 0.05 mg/ml. Bacterial suspensions at high density seem to contain enough EPS to protect bacterial cells from the lethal dilution effect."

Goto (1992) later notes, "Within canker lesions, cells of the bacterium are usually buried in a thick matrix of EPS. These facts imply that the bacterium can survive for fairly long periods if the EPS around them dry and are left intact." Goto (1992) specifically notes that without EPS the bacteria are short lived and even rain events may dilute the bacterial solution (specifically, the EPS concentration) enough that it results in the death of the disease agent.

Fulton and Bowman (1929) reported a series of studies on *Pseudomonas citri* [Syn. *Xanthomonas axonopodis* pv. *citri*] and reported that wounding needed to be done with care not to cut oil glands in order for infection to occur. They noted, "In the case of young fruit it was found that the puncturing instruments ruptured some of the oil glands. The exuding oil had a tendency to injure a portion of the adjacent tissue and to interfere with a normal infection reaction." The authors then infected fruit with *Pseudomonas citri* by making a large number of punctures singly with a needle either directly into the oil glands, or elsewhere on the same fruit and taking care to avoid the oil glands. The puncture wounds that avoided the oil glands developed significantly more citrus canker lesions (8-80% canker lesions development) as opposed to wounds made directly into oil glands (0-8% canker lesion development) (Fulton and Bowman, 1929). It would be unlikely that naturally occurring wounds would include extreme care to cause wounding with the accuracy of a needle to avoid rupturing oil glands. Goto (1992)

also suggests using sharp needles for inoculation, "a shallow prick reaching only to the mesophyll…", and notes that larger wounds such as holes through leaves may result in necrosis and unsuccessful infection. Fulton and Bowman (1929) also noted that infection only occurred if the wound stayed moist until the time of inoculation. Wounds that were allowed to dry and were inoculated after 26 hours did not result in successful infection. That is, infections occurred only when oil glands were avoided and inoculum was applied within 26 hours. Infections dropped dramatically if fruit was allowed to dry for more than 1 hour (observations made with less than 1 hour drying time had 2-70% infection whereas observations at 8 hours showed 0-6% infection). The results using eight varieties showed ten sets of observations (different varieties) with 0% infection after 8 hours and three sets with 2-6% infection.

Fulton and Bowman (1929) noted, "Fully ripe fruit inoculated through wounds after removal from the tree did not give evidence of any definite increase of the canker organism." In the absence of infection; Fulton and Bowman (1929) were able to culture canker from a single inoculated grapefruit 74 days after inoculation. However, common rots such as *Penicillium* inhibited or killed the bacteria if the fruit were not protected from such infection.

A review of the Fulton and Bowman (1929) experiments leads us to conclude that wounds may be a significant factor in immature fruit. For mature fruit, wounds that are made with care to avoid oil glands and that do not become infected by other organisms (such as *Penicillium*) can be a reservoir of the canker organism for extended periods (74 days reported) (Fulton and Bowman, 1929). A scenario where carefully inoculated fruit remains protected from other organisms is extremely unlikely to exist in nature and is therefore an unlikely pathway for disease spread.

Epiphytic[6] populations of Xac may aid in pathogen dispersal, but the bacteria only survives for a limited amount of time; 8-72 hours in sun and shade (Goto, 1969; Goto, 1962). No variation of survival has been detected for epiphytic Xac bacteria on varying plant or inorganic material. Gottwald examined epiphytic bacteria survival on metal, wood, plastic, cloth, leather, and grass; he did not detect Xac on any surface tested after 72 hours (Workshop Transcript, 2000). In one study, leaves inoculated with culture-grown bacteria displayed a significant population decline (3-5 orders of magnitude) in the first 24 hours following inoculation, but bacteria persisted in low numbers ($<10^4$ cfu/leaf) for up to 5 days (Timmer *et al.*, 1996). In a laboratory study inoculating unwounded leaves via air pressure, bacteria concentrations of less than 10^4 cells per ml were unable to cause infection (Gottwald and Graham, 1992). Wounded leaves are more susceptible to infection and may only require a minimum of 10^2 cells per ml (Goto *et al.*, 1978). Epiphytic bacteria do not multiply in water on leaf surfaces or on dry leaves (Timmer *et al.*, 1991; Timmer *et al.*, 1996). There is therefore a very limited window of time when epiphytic population will pose an epidemiological threat.

Populations of epiphytic Xac populations appear to vary greatly in the field. Timmer *et al.* (1996) examined epiphytic populations of Xac on Duncan grapefruit leaves from severely affected symptomatic and asymptomatic seedlings (placed between symptomatic seedlings). All seedlings had mature fully expanded leaves, which were resistant to new infection. All plants were irrigated by overhead sprinklers. The leaves of both plants were swabbed and the bacteria

[6] The term "epiphytic" in this document is used to describe Xac bacteria existing on any exposed plant surface without causing infection (Agrios, 1988).

were incubated on plates for three days. Symptomatic leaves, on average, harbored 10^5 cfu/leaf[7], but could be as high as 10^7 cfu/leaf immediately following a period of rain. Epiphytic populations on asymptomatic leaves were highly variable ranging from less then 10^1 to 10^5 cfu/leaf (Timmer *et al.*, 1996).

Rybak and Canteros (2001) examined population levels of epiphytic Xac on healthy asymptomatic leaves and harvested fruit from groves with and without citrus canker control programs; displaying low and high disease incidence in the field. Xac was detected by washing healthy leaves and fruit, and then plating and infiltrating susceptible grapefruit leaves with the resultant. The amount of Xac detected on healthy leaves and fruit varied in relation to disease incidence and control program existence. Xac levels were undetectable in low disease incidence sprayed groves, while unsprayed high disease groves yielded to 0-10^6 cells/leave or fruit (Rybak and Canteros, 2001). Forty-six asymptomatic fruit collected from naturally infected citrus groves in Uruguay and Argentina, and assayed for epiphytic Xac, yielded on average 2.0 x 10^3 cfu/fruit (Verdier *et al*., unpublished).

Spread Potential

Citrus canker spread from an initial 50 square miles to 1,265 square miles in the first five years following its most recent detection in Florida. The epidemic centered on the Miami area (Gottwald *et al.*, 2001). The rate of spread may be attributed to suitable environmental conditions that included storms with high winds and wind-driven rain on a regular basis, as well as limited management of the disease. For example, a thunderstorm in 1989 spread inoculum resulting in the establishment of four new disease foci, 230 to 810 m from the infected source trees (Gottwald *et al.,* 1992a; Graham *et al.*, 2004b). The disease is less likely to develop and easier to eradicate in areas where high winds, storms, high humidity and suitable temperatures are less frequent (Graham and Gottwald, 1991).

Borchert *et al.* (2007) developed a citrus canker spread model using the North Carolina State University APHIS Plant Pest Forecast System (NAPPFAST) to identify areas where citrus canker could become established in the major citrus producing regions of the United States. The study used three approaches to analyze the likelihood of establishment: i) spread events for selected stations based on hourly rain and wind data (Figure 2); ii) infection model using daily climate data at a 10-km^2 resolution (Figure 3); and iii) analysis of average monthly climate data at a 55 km^2 resolution. The study concluded that the "… climate in Florida is highly favorable for citrus canker in terms of predicted spread, favorable days for infection, and average temperature. The Louisiana and Texas citrus growing areas have temperature, favorable days for infection and wind-rain conditions conducive for moderate to high Xac disease intensity, with conditions in Louisiana more conducive than Texas. The Arizona citrus growing area has temperature conditions conducive for Xac infection, but a low annual precipitation, low favorable days for infection, and few spread events, which results in low potential disease intensity. The California citrus growing areas have fewer months of temperatures conducive for Xac infection than the other citrus growing areas in the United States. California also had the lowest number of wind-rain events and infection days with the exception of Arizona. In California, these events occur predominantly during the winter, while the warm summer months

[7] "cfu" refers to colony forming units.

are dry, accounting for less than 5% of the annual precipitation. The combination of fewer months conducive for infection and lower frequency of spread events, especially when trees are more susceptible (i.e. summer) results in lower potential disease intensity of Xac in California (Borchert *et al.,* 2007)."

Long distance spread of the pathogen can occur via the movement of infected planting and propagating material, such as budwood, 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, 2002; 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 a potential means of long distance pathogen spread; however, there is no authenticated record of this being related to the epidemiology of the disease (Smith *et al.*, 1997), despite a long global history of commercial trade in citrus fruit. Likewise, infected culled fruit and processed fruit pulp have also been identified as possibly facilitating long-distance pathogen spread. The likelihood of this occurring is unknown (Smith *et al.*, 1997). There is no evidence 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; Anonymous, 2005a; Anonymous, 2005b).

Disease Management

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 and Leite, 2004a; Muraro *et al.*, 2001; Das, 2003; Kuhara, 1978; Koizumi, 1977; Medina-Urrutia and Stapleton, 1985; Timmer, 1988). Additional copper fungicide sprays are often necessary to avoid symptoms in generally infected areas because the initial spray usually wears or washes off reducing its efficacy in limiting disease spread potential (Stall *et al.*, 1980; Leite and Mohan, 1990; Gottwald *et al.*, 2002a).

The effectiveness of copper-based sprays alone is dependent on the susceptibility of the citrus cultivar to Xac, and the frequency of sprays (Kuhara, 1978; Leite *et al.*, 1987; Goto, 1992). Rainfall lowers the effectiveness of copper sprays (Serizawa and Inoue, 1978; 1982). In Japan, susceptible hosts are often sprayed 5 to 7 times, while moderately susceptible cultivars are only sprayed 4 times throughout the growing season. Five to six copper sprays during the highly susceptible rainy season in Brazil results in a decrease in disease incidence of 90% in moderately susceptible cultivars (Leite *et al.*, 1987). In addition, the age of the citrus tree, environmental conditions, and the application of other control methods may alter the effectiveness of copper sprays (Leite, 2000). Graham and Gottwald (1991) state "in the absence of windbreaks, copper applied at 1-month intervals slightly reduced canker spread during wind-blown rain events."

However, they found that copper applications did not affect disease incidence when used in conjunction with windbreaks and applied at one-month intervals.

Management of Xac may be aided by forecasting systems that include weather monitoring that may be used to prevent epidemics 1 to 2 months in advance. Weather monitoring systems may examine the number of over-wintered lesions on angular shoots, frequency and amount of precipitation, number of windy days, temperature, and the concentration of Xac in rainwater running on the leaf surface. Advance notice of Xac epidemics allows growers to take subsequent steps to effectively tailor their field control programs (Goto, 1992). Goto (1992) goes on to review specific management practices that allow highly effective production [Japan exports citrus] even when canker is present. It is also noted that production conditions for citrus in Japan include very high humidity, warm temperatures and frequent high winds [typhoons are not rare in the region].

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). In vitro tests exposing Xac to as little as 0.1 ppm Cl eliminated all Xac bacteria (Stapleton, 1987). 200 ppm Cl applied for 2 minutes to fruit collected from the field showed that natural bacterial populations were lowered by 77-99%; no Xac was recovered post-treatment, although the authors did not assess the level of Xac (if any) that was present on the fruit prior to treatment (Stapleton, 1986). Similar results were obtained using chlorine and/or sodium orthophenylphenate (SOPP) treatments; fruit with $<10^7$ cells/ml of Xac dipped in 100 ppm chlorine yielded no detectable levels of bacteria (Obata, 1969). Studies performed in Argentina on the effectiveness of sodium hypochlorite on mature symptomless fruit artificially contaminated with Xac showed that sodium hypochlorite levels as low as 8 ppm were effective in eliminating epiphytic or surface bacteria from the fruit (Canteros, undated). Successive treatments of sodium hypochlorite and sodium orthophenylphenate (SOPP), common in many packinghouse procedures, are highly effective in eliminating epiphytic populations of Xac. Asymptomatic fruit naturally and artificially contaminated with Xac subjected to the above treatments eliminated 99.5% of epiphytic Xac bacteria. Whereas, there was not 100% destruction of the bacteria, it was not possible to culture the surviving bacteria on susceptible leaf tissue. The bacteria could only be recovered in artificial growing media (Verdier *et al*., unpublished). Canteros *et al*. (2001) showed that the combined treatments of sodium hypochlorite and SOPP completely eliminated Xac from fruit and leaf surfaces.

Verdier *et al*. (unpublished) found that treatments of infested fruit (naturally and artificially infested fruit) using commercial practices (chlorine wash, SOPP wash, fungicide application, and waxing) resulted in 99.5% control of disease. Whereas they were able to culture canker bacteria in artificial growing media (XOS) in a small proportion (0.5%) of the treated fruit, they were not able to observe disease development in actual plant tissue even when using a highly susceptible variety. Similar treatments of commercial fruit would result in very effective control of the disease and greatly reduce the likelihood that the pathogen could spread. Commercial fruit is unlikely to be as highly infested as the fruit used in the Verdier *et al.* experiment (67%) where a high level of infestation was obtained from obviously diseased, symptomatic trees after suitable moisture conditions had been observed immediately prior to fruit collection.

Commercial applications in a packinghouse have greater variability than laboratory studies which are dependent on controlling a number of factors, including the design for treatment application and whether sodium hypochlorite, SOPP, or both, are used. Brown and Schubert (1987) note that the effectiveness of chlorine is compromised by organic compounds whereas the effectiveness of SOPP is not. Proper design, application, and oversight of the treatment is necessary to account for operational aspects of specific commercial situations; and to ensure efficacy, as well as reduce variability associated with treatment.

Epiphytic populations of Xac that might survive post-harvest treatment would not be expected to survive on the surface of fruit for longer than 72 hours (Goto, 1969; Workshop Transcript, 2000; Timmer *et al.*, 1991), and would not be expected to multiply. Epiphytic bacteria do not multiply in water on leaf surfaces, or on dry leaves (Timmer *et al.*, 1991; Timmer *et al*., 1996).

Phytosanitary Evidence Regarding Asymptomatic Fruit as a Pathway[8]

The information above reviewed the background related to the biology of the disease and its management. This section reviews the risks associated with commercially produced citrus fruit. Here we evaluate how the evidence informs specific components of the pathway represented by domestic and international trade in citrus fruit. As appropriate, we identify specific evidence associated a given element of the pathway and articulate our findings based on our interpretation of that evidence.

Previous analyses have assessed the likelihood of introducing the pathogen from citrus fruit (Schubert, *et al.*, 1999; USDA, 1995; USDA, 1997; USDA, 2002). Assuming that it may be possible for fruit to serve as a pathway for introduction of the disease, several events have been identified as necessary for the pathogen to enter and establish in a new area on commercial fruit.

These events include:

(1) Infected/contaminated fruit are harvested;
(2) Inoculum associated with fruit survives the packing/treatment process;
(3) Inoculum associated with fruit survives shipment;
(4) Fruit with inoculum go to an area with conditions suitable for infection; and
(5) Inoculum encounters a suitable host and conditions for disease development.

<u>Event 1: Infected or contaminated fruit are harvested</u>

This event is assumed to be likely if fruit originates in areas where the disease occurs. The magnitude of the hazard depends 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

[8] The list of scientific literature that informs the preceding section from the present discussion is separated to make it clear which items amongst all those reviewed were considered key in reaching the conclusions described in this section. Thus, the scientific evidence described at the end of the document as Literature Cited and Bibliography sections.

with lesions, injuries or blemishes, or it may be epiphytic (surface contamination). Research does not indicate the existence of endophytic infection inside the fruit or any plant part that does not develop symptoms. The prevalence of infected or contaminated fruit will primarily depend on the variety, environmental conditions, and field treatment regimes.

Citrus canker is mainly a leaf spotting and fruit blemishing disease. The disease produces obvious and characteristic symptoms on fruit, except when uncharacteristic lesions may be associated with injuries (Gottwald and Graham, 2000; Pruvost *et al.,* 2002; Timmer *et al.,* 2005). Typical lesions from natural infection are unlikely to occur on mature fruit since the natural infection of healthy uninjured fruit occurs as the young fruit is enlarging (Civerolo, 1984; Graham *et al.*, 1992b; Verniere *et al.*, 2003; Graham and Leite, 2004a). Infection after the 90-120 day period of fruit enlargement is therefore associated with injuries, and may not result in typical symptoms. Mature fruit exposed to the bacteria through an injury would not be expected to express symptoms although it is possible that injured fruit could harbor the bacteria. Fruit that is mature (not expanding) and is free of 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 the location of the canker lesions (Schubert *et al.*, 2001). Fruit and leaves 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). Timmer *et al.* (1991) inoculated grapefruit and Swingle citrumelo leaves with *Xanthomonas camperstris* pv. *citri* in the field, and then collected the leaves at 14, 21, and 49 days after inoculation to assess bacteria concentrations within the active lesions. Fourteen day old lesions produced significantly more bacteria than 49 day old lesions. Young leaves (4-6 weeks old) and mature leaves (4-6 months old) naturally infected with *X. c.* pv. *citri* were removed from the infected tree and assessed in the lab. Bacterial populations within the lesions were closely correlated with lesion age. Young leaves exuded ~10^6 cells/ml, while older lesions exuded ~10^3 cells/ml. After a 48-hour period, all samples exuded fewer then 10^2 cells/ml (Timmer *et al.*, 1991). Fruit and leaves removed from trees sustain bacterial populations in lesions for 1-2 months in the presence of antagonistic microorganisms (Schubert *et al.*, 2001). Diseased leaves removed from trees and placed on the soil surface may harbor bacteria populations for 90 to 120 days, with no detectable bacteria after 120 days (Graham *et al.,* 1987). Xac populations within the lesions of infected fruit decline after harvest (Civerolo, 1981; Koizumi, 1972).

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.* (1996) 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 within the lesions of infected fruit after harvest (Civerolo, 1981). Researchers in Brazil sprayed asymptomatic fruit, removed from trees, with a bacterial suspension of 10^6 cfu/ml; this resulted in non-recovery of inoculated bacteria after 5 days at room temperature under lab conditions (Belasque and Neto, 2000). The survival of epiphytic

Xac populations on asymptomatic fruit, under natural conditions, would be expected to be further reduced due to harsher environmental conditions.

In commercial operations, diseased, damaged, disfigured, and blemished fruits are culled in the field and packinghouse. Current programs in Argentina have demonstrated that culling of symptomatic citrus canker fruit is highly effective in packinghouse operations. For example, trays of fruit known to contain either 1% or 3% of symptomatic fruit were visually inspected at three stages throughout the packing process, resulting in extremely low (near zero) number of symptomatic, injured or blemished fruit reaching the packing bench, and zero symptomatic fruit packed in boxes (Ploper *et al.*, 2004). Because the survival of bacteria on non-attached citrus fruit is significantly reduced (Koizumi, 1972), the likelihood of citrus canker spread through bacteria on harvested commercial fruit is very low (Graham *et al.*, 1992b). No infection was observed on citrus fruits artificially inoculated with *Xanthomonas axonopodis* pv. *citri*, even under ideal environmental conditions (Civerolo, personal communication on unpublished research).

<u>Conclusions for the first event:</u>

1.1. Xac is present in groves with active infections, or likely to be present in nearby groves from which the bacteria may be introduced by wind-driven rain.

Primary evidence:

- Bacteria are dispersed within trees, and from tree to tree through wind-driven rain. When wind-driven rain 8 m/sec or greater occurs it aids the penetration of bacteria through stomatal pores (Timmer *et al.*, 2000), therefore increasing the likelihood of infection in unwounded tissue.
- The bacteria can disperse from 32 meters to several miles (Stall *et al.*, 1980; Gottwald *et al.*, 1988, 1992, 2002b; Timmer *et al.*, 2000).
- In Florida, the pathogen is found to spread 1900 ft from the source, 95% of the time (Gottwald, 2002a; Gottwald *et al.*, 2001; Compton and Fagan, 2000; McElroy, 2000).

1.2. Infected fruits are likely to be culled due to the presence of lesions or injuries.

Primary evidence:

- Citrus canker is mainly a leaf spotting and fruit blemishing disease. Symptoms of citrus canker on fruit are visually obvious (Brunings & Gabriel, 2003).
- Mature (not expanding) asymptomatic fruit without injuries or blemishes are not known to develop symptoms (Graham, *et al.*, 1992b; Goto, 1992).
- Commercial operations can be highly effective in removing diseased, damaged, disfigured, and blemished fruit through a combination of culling in both the field and packinghouse (Ploper *et al.*, 2004).

1.3. The epiphytic presence of Xac on fruit does not have a significant role in pathogen spread. Xac in symptomless, mature fruit produced using commercial practices is likely to be epiphytic and labile.

Primary evidence:

- Epiphytic populations of Xac on citrus tissue drop 3-5 orders of magnitude to $<10^4$ cfu/leaf in 24 hours during experimental testing (Timmer *et al.*, 1996). Timmer *et al.* (1996) 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."
- Epiphytic populations of Xac only survives for a limited amount of time, 8-72 hours, in sun and shade locations (Goto, 1969; Goto, 1962), although one study showed populations of $<10^4$ cfu/leaf present for up to five days post artificial inoculation (Timmer *et al.*, 1996). Epiphytic bacteria do not multiply in water on leaf surfaces or on dry leaves (Timmer *et al.*, 1991; Timmer, 1996).
- Researchers in Brazil sprayed asymptomatic fruit with a bacterial suspension of 10^6 cfu/ml, resulting in non-recovery of inoculated bacteria after 5 days at room temperature under lab conditions (Belasque and Neto, 2000).

<u>Event 2: Inoculum associated with 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 packinghouse where it is subjected to one or more visual inspections. Field culling will remove a high percentage of obviously symptomatic and blemished or injured fruit prior to arrival at the packinghouse. Visual and/or mechanical inspection at the packinghouse further reduce the possibility that symptomatic, injured, or blemished fruit, as well as misshapen, off-color, damaged, and otherwise low quality fruit, will be packed. The efficacy of these culling processes varies depending on the facility and system, but APHIS' experience with these processes in the United States, Argentina, Japan, and other countries show that it is highly effective in removing suspect fruit. For example, citrus fruit harvested in Argentina for export to the European Union have <1% symptomatic fruit from the field. Culling procedures in Argentina are extremely effective in removing symptomatic fruit, resulting in extremely low numbers (near 0) of symptomatic fruit reaching the packing bench, and zero symptomatic fruit packed into boxes (Ploper *et al.*, 2004).

Typically, citrus fruit is washed when it enters the packinghouse as a normal part of the packing process. This washing usually includes mechanical brushing and detergent (at least), which will remove organic and other contaminants (such as soil) (Schubert, *et al.*, 1999). Brushing increases the exposure of Xac to detergents or other toxins (Brown and Shubert, 1987). The drying process, *i.e.*, hot air at 58 °C for 2.5 minutes, after washing further reduces epiphytic Xac populations (Schubert *et al.*, 1999).

When the washing process includes a disinfectant, such as 200 ppm chlorine or sodium orthophenylphenate (SOPP), Xac populations are significantly reduced to epidemiologically insignificant or undetectable levels (Brown and Shubert, 1987; Graham and Gottwald, 1991; Obata, 1969). The post-harvest treatments, such as chlorine or SOPP, guarantee the complete eradication of epiphytic Xac on fruits without symptoms (Canteros *et al.*, 2001; Canteros, 2004; Canteros, undated).

The use of 200 ppm Cl for two minutes reduces natural bacterial populations on citrus fruits from 77-99% (Stapleton, 1986). The estimated reduction in bacterial populations on the surface of citrus fruits brushed, washed, and disinfected with Cl or SOPP is between 3 and 7 orders of magnitude, according to research cited above. According to research by Verdier *et al*. (unpublished) (Anonymous, 2005b), bacterial populations on artificially infested fruits were $1.7x10^3$ cfu/ml and 39.4 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, detected through plating on artificial growing media. No growth or disease developed on tests using susceptible leaf tissue. Bacterial populations used for artificial inoculation of fruit are at extremely high levels typically never found in natural conditions (~43 times higher than naturally infected fruit) (Verdier *et al*., unpublished). Such levels of bacterial presence, as used in these experiments, is considered "high" because those levels are consistent with every tree in a grove being infected (as all fruit in the experiments are inoculated or exposed to Xac). That very high level of disease incidence is not likely to be found in any commercial grove anywhere because the grove would not be productive and the fruit would not be marketable. Studies preformed in Argentina on the effectiveness of sodium hypochlorite on mature symptom-less fruit, artificially inoculated with Xac, showed that sodium hypochlorite levels as low as 8 ppm were effective in eliminating epiphytic or surface bacteria from fruit (Canteros, undated). If small epiphytic population of Xac did survive the post-harvest treatment it would not survive on the surface of the fruit for long (Goto, 1969; Workshop Transcript, 2000; Timmer *et al.*, 1991). Epiphytic bacteria do not multiply in water on leaf surfaces or on dry leaves (Timmer *et al.*, 1991; Timmer *et al.*, 1996).

<u>Conclusions for the second event:</u>

2.1. Symptomatic fruit are highly unlikely to pass through the packing process.

Primary evidence:

- Visual and/or mechanical inspections in the packinghouse remove symptomatic or blemished fruit, as well as misshapen, off-color, damaged and low quality fruit.

2.2. Standard packinghouse procedures and post-harvest treatments prescribed by the systems approach will remove and/or devitalize ephiphytic populations of the pathogen.

Primary evidence:

- Washing and brushing will reduce epiphytic bacterial populations and the drying process (with hot air at 58 °C for 2.5 minutes) 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 epidemiologically insignificant or undetectable levels (Brown and Shubert, 1987; Obata, 1969; Canteros, undated).
- Post-harvest treatments, such as chlorine and SOPP, common in many packinghouse procedures, guarantee the complete eradication of epiphytic Xac on fruits without symptoms (Canteros, 2004; Canteros *et al.*, 2001).
- The use of 200 ppm Cl for two minutes reduces natural bacterial populations on citrus fruits from 77-99% (Stapleton, 1986).
- Even if small epiphytic populations of Xac did survive post-harvest treatment, they

would not survive on the surface of the fruit longer than 72 hours (Goto, 1969; Workshop Transcript, 2000; Timmer *et al.*, 1991). Epiphytic bacteria do not multiply in water, on leaf surfaces or on dry leaves (Timmer *et al.*, 1991; Timmer *et al.*, 1996).

- Phytosanitary safeguards associated with the systems approach are applied to prevent epiphytic recontamination by the pathogen during packing and handling.
- Bacterial-host interaction results in the production of specific fluids (e.g., extracellular polysaccharides, EPS) inside lesions that improve the stability and survival of these bacteria. However, when diluted (as is expected to happen during multiple washes, bactericidal treatments, and dips) their stability drops and bacteria are more likely to die (Goto, 1992).

Event 3: Inoculum associated with fruit survives shipment

The third event is concerned with the survival of inoculum during shipping. Bacteria that may survive the packing process have not been shown to occur on the surface of citrus fruit or under the wax coating applied to the fruit. Any surviving bacteria are not likely 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 (Anonymous, 2005b), and there is no evidence of epiphytic growth by Xac (Brunings & Gabriel, 2003).

The multiplication of Xac bacteria associated with lesions is closely related to lesion expansion. In expanding lesions, Xac bacteria multiply abundantly, but as lesion expansion ceases, bacteria multiplication noticeably decreases (Koizumi and Kuhara, 1982). As lesions expand, with the exception of the early stages of lesion development, the cells in the center of lesion disintegrate. In the late stages of lesion expansion, bacteria multiplication becomes inhibited in the peripheral area of the lesion (Koizumi, 1977c). Bacteria survive 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). Xac bacteria do not increase in number on fruit once the fruit is removed from the tree, but rather populations decline within the lesions of infected fruit following harvest (Civerolo, 1981; Koizumi, 1972).

Conclusions for the third event:

3.1. Bacteria that survive the packing process will have a high rate of mortality during shipping. Primary evidence:

- Epiphytic populations of Xac on citrus tissue drop 3-5 orders of magnitude to $<10^4$ cfu/leaf in 24 hours during experimental testing (Timmer *et al.*, 1996). Timmer *et al.* (1996) 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." There is evidence (e.g., Goto, 1992) that bacterial-host interaction results in the production of specific fluids (such as Extracellular Polysaccharides) inside lesions that improve the stability and survival of these bacteria. However, when they are diluted (as is expected to happen during multiple washes, bactericidal treatments and dips) their stability drops and bacteria are more likely to die (Goto, 1992).
- Epiphytic populations of Xac only survive for a limited amount of time, 8-72 hours, in

sun and shade locations (Goto, 1969; Goto, 1962), although one study showed populations of $<10^4$ cfu/leaf present for up to 5 days post artificial inoculation (Timmer *et al.*, 1996). Epiphytic bacteria do not multiply in water on leaf surfaces or on dry leaves (Timmer *et al.*, 1991; Timmer *et al.*, 1996).

- Researchers in Brazil sprayed asymptomatic fruit with a bacterial suspension of 10^6 cfu/ml, resulting in non-recovery of inoculated bacteria after 5 days at room temperature under lab conditions (Belasque and Neto, 2000). The survival of epiphytic Xac populations on asymptomatic fruit (under natural conditions) would be expected to be further reduced due to harsher environmental conditions.
- Populations rapidly decline within the lesions of infected fruit after harvest (Civerolo, 1981; Koizumi, 1972).
- Bacterial populations within lesions on removed plant material rapidly decline to epidemiologically insignificant levels. After 48 hours, lesions exuded fewer then 10^2 cells/ml (Timmer *et al.*, 1991).

3.2. Bacteria that survive on the fruit's surface or in lesions/injuries associated with fruit, after post-harvest treatment, will not multiply or cause disease development in treated fruit.

Primary evidence:

- An epiphytic growth stage for citrus canker has not been observed (Brunings & Gabriel, 2003).
- There is no evidence that infected fruit with lesions or asymptomatic fruit are epidemiologically significant with respect to the initiation of new infections (Jetter *et al.*, 2000; Canteros, 2004; Anonymous 2005a,b).

<u>Event 4: Fruit with inoculum go to an area with conditions suitable for infection</u>

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, but it is distributed according to market demand. Demographics derived from United States Census data may be useful in predicting the distribution of imported and domestically grown citrus fruit by indicating population centers where demand is greatest. Three of the four most populous states in the United States, Florida, Texas, and California, are in the southern tier of the United States where the climate most closely resembles the native climates for Xac (U.S. Census, 2002). These three states account for approximately 25% of the total U.S. population (U.S. Census, 2002. If we assume that citrus is proportionally distributed across the United States, in accordance with population, then it is reasonable to assume that some fruit will be shipped to these states; however, only a small portion of each state actually produces citrus, and an even smaller portion has a climate suitable for Xac disease development. Similar analyses may apply to other countries where citrus is grown.

<u>Conclusions for the fourth event</u>:

4.1. Although shipment of imported and domestically grown infected fruit to a suitable habitat is possible, the fraction that would be shipped to a suitable habitat is small. The fraction that would reach a suitable host is smaller.

Primary evidence:

- Only a relatively small proportion of the citrus growing areas in the United States are at risk, because suitable conditions for the disease to develop occur mainly in Florida (e.g., Borchert *et al.,* 2007).
- Citrus canker is primarily a disease of tropical and subtropical regions; according to Borchert *et al.* (2007) potential disease intensity in the United States would be affected by the frequency of spread events, temperatures, timing of precipitation, host susceptibility, and the citrus leafminer.
- The disease is less likely to develop in areas where warm rainy conditions are less frequent (Graham and Gottwald, 1991).
- Of the citrus producing areas in the United States Arizona has the lowest risk for Xac establishment due to the low number of spread events and dry conditions. California also has a low risk for Xac establishment because favorable events for Xac establishment occur predominantly during the winter season when precipitation is greatest, but temperatures are less conducive for infection and citrus growth. Texas and Louisiana are more conducive to Xac establishment as favorable events occur regularly throughout the year as does precipitation and monthly temperatures conducive for infection (Borchert *et al.,* 2007).

<u>Event 5: Inoculum encounters a suitable host and conditions for disease development</u>

The fifth event is that the Xac inoculum on imported or domestically grown fruit encounters a suitable host within the suitable habitat. Furthermore, the host must be at a stage susceptible to canker, with proper environmental conditions for disease to occur. Transmission scenarios include but are not limited to:

1. A host plant caretaker eats a canker-infected fruit and trims citrus trees thereafter. This mode of transmission is unlikely because the acidic citrus juice is bactericidal (Schubert *et al.*, 1999). The chance for manual vectoring of the canker bacteria to a new host is considered extremely remote (by "extremely remote" we mean less likely than baseline exposure, or that constituted by exposure through existing non-regulated pathways).

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). Rapid decomposition dramatically reduces the chance of transmission over time (Goto *et al.*, 1978). For bacteria to move unassisted from the ground, a compost pile, or trash can to susceptible tissue on branches, fruit, or leaves, rain and wind must be available (Gottwald *et al.,* 2001).

3. An infected or contaminated citrus fruit comes into direct contact with a susceptible citrus tree and causes infection; however, this scenario is unlikely because the contaminated fruit would need to possess enough bacteria to cause infection on susceptible trees. Xac populations on processed fruit would unlikely contain large enough populations for infection due to the following reasons:

1) The fruit would have undergone commercial packinghouse procedures, such as washing, post-harvest treatment of SOPP, CL, or SOPP and Cl, and waxing. All of these components would result in a significant reduction in the epiphytic population present on the asymptomatic citrus fruit (Schubert *et al.*, 1999; Brown and Shubert, 1987; Graham and Gottwald, 1991; Obata 1969; Canteros, undated; Canteros, 2004; Canteros *et al.*, 2001; Stapleton, 1986).

2) Epiphytic bacteria of Xac do not multiply (Timmer *et al.*, 1991; Timmer *et al.*, 1996), and only survive for a limited amount of time on fruit surfaces (Goto, 1969; Workshop Transcript, 2000; Timmer *et al.*, 1991; Timmer *et al.*, 1996). Any epiphytic Xac bacteria populations that do survive the packing process continue to decrease in population size over time (*i.e.,* during shipment).

The development of disease requires that the inoculum successfully infect susceptible hosts, assuming the inoculum is available. The following must occur for disease development to occur (van der Plank, 1963; Agrios, 1988):

- 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 sufficient force (i.e. wind) must be present to drive the bacteria into natural openings, or wounds must be present.
- The causal organism must be in a stage that can cause disease onset
- Enough time must elapse for key interactions to occur

<u>Conclusions for fifth event:</u>

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

Primary evidence:

- Known hosts of Xac are almost exclusively in the Rutaceae, particularly *Citrus* spp. (Smith *et al.*, 1997).
- The majority of leaf infection occurs during enlargement (Civerolo, 1984), over a short time interval of susceptibility with the majority of infection occurring within the first six weeks (Timmer *et al.*, 2000).
- Fruits are susceptible to infection from petal fall to 90-120 days thereafter. Fruit can be exposed to several infection cycles (Schubert *et al.*, 2001; Civerolo, 1984; Gottwald *et al.*, 2002a; Timmer *et al.*, 2000). Fruits 2-6 cm in diameter are highly susceptible to infection; however, susceptibility reduces as fruit develops (Civerolo, 1984; Graham *et al.*, 1992b; Verniere *et al.*, 2003; Graham et. al., 2004a).
- 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 to 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 enhanced by wind-driven rain; in which wind speeds reach an excess of 8 m/sec to create enough force to drive

inoculum infested water into the stomata (Gottwald *et al.*, 2002a; Graham *et al.,* 2004b). Infection is likely when high moisture and wind conditions occur in conjunction with the presence of sufficient inoculum and susceptible hosts.

- There is no authenticated record for diseased fruit playing a role in the epidemiology of citrus canker disease (Smith *et al.*, 1997), despite the existence of global commerce in citrus fruit.
- 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; Anonymous, 2005a,b).

Observations about Baseline Exposure ("de minimis risk" considerations)

Other analyses have acknowledged 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 United States undetected, or is not inspected, is unknown. However, numerous years of interception data show that Xac has been intercepted more than 1,900 times, while the genus *Xanthomonas* has been intercepted over 13,000 times at U.S. ports-of-entry mainly associated with passenger baggage (APHIS PIN309). It has been previously observed that the number of interceptions represent a fraction of the total that actually enters the United States (Meissner et al., 2003).

Based on these studies and the constraints of port inspectors to inspect baggage, the entry of citrus canker-infected fruit thousands of times in the past is conceivable. It is reasonable to assume that most of the fruit that is moved illegally has probably not been subject to a "systems approach" (citrus in baggage is frequently from dooryards). There is no evidence that mature citrus fruit can act as a pathway for the transmission of the pathogen or that Xac outbreaks have been attributed to unauthorized imports. Such movement has not been associated with an outbreak of the disease anywhere in the world.

Empirical data from programmatic experiences, in addition to interceptions, demonstrate that even with a high frequency of unauthorized citrus fruit imports, outbreaks linked to fruit have never been observed. Authorized imports, based on a systems approach, demonstrate 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 not an important source of inoculum (Timmer *et al.*, 1996).

Summary

A systems approach that controls infection in the grove, excludes contaminants and fruit with citrus canker symptoms or injuries from packing, and uses an effective surface disinfection treatment is highly effective in mitigating the likelihood of fruit as a pathway for the introduction and spread of citrus canker.

The wash treatment eliminates epiphytic contamination as a hazard when done correctly. The remaining hazard is the small possibility that symptomatic fruit escape culling. In this case, the fruit would be expected to have a very low inoculum level after harvest and treatment (*i.e.*, being

washed, treated, and waxed). Any inoculum remaining or produced afterward would rapidly lose viability. Assuming that an infected fruit escapes inspection 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; without the fruit first decaying or being otherwise destroyed by the process of consumption and discarding of peels into the garbage or compost heaps – all of which contributes to the putrefaction of the substrate. It is extremely unlikely that fruit will encounter the specific range of conditions necessary to infect another plant.

These points consistently argue that asymptomatic fruit is not epidemiologically significant as a pathway for introducing citrus canker when produced under the conditions of a systems approach consistent with the assumptions of this analysis. The evidence is not as conclusive regarding symptomatic fruit, but because the symptoms are characteristic and obvious, culling can be highly effective as a means to reduce the prevalence of symptomatic fruit to very low levels. Considering the very low number of symptomatic fruit that might escape detection, the limited areas where appropriate hosts and conditions exist, and the very low probability for conditions suitable for establishment to occur, the introduction of the disease via this pathway is highly unlikely.

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.) and despite the evidence from a long history of global trade in citrus fruit. There is no scientific evidence that an outbreak can originate from asymptomatic commercial fruit (Civerolo, 1997). Experiments suggest that when conditions are suitable, the likelihood of transmitting citrus canker from Xac in incipient citrus canker lesions or from epiphytic Xac on citrus fruit is low (E. Civerolo, personal communication on unpublished research). Any other experiments not documented in scientific literature needs to be formally summarized and communicated to APHIS.

Further research could strengthen the evidence surrounding the epidemiological significance (or lack there of) of Xac on symptomatic and asymptomatic commercial citrus fruit. Despite the presence of citrus canker in the United States and elsewhere, research gaps continue to 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)?

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

Issue: How effective are different products at reducing the biological activity of bacteria in lesions (*i.e.*, what is the efficacy of various post-harvest treatments (*e.g.*, SOPP, chlorine, etc) at rendering symptomatic fruit epidemiologically insignificant)?

Importance: Chlorine is a common standard, but it is also unstable and needs constant monitoring and replacement. 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?

Importance: Infected groves or partially infected groves may be part of an exporting area. It is 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?

Importance: 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.

Issue: How long after post-harvest treatment can Xac be recovered from asymptomatic fruit.

Importance: The canker bacteria are not very long-lived outside of suitable conditions. As fruit is treated and dried and then stored, there may be sufficient mortality (even in fruit with lesions) to render such fruit epidemiologically insignificant (not a pathway) because of the time this fruit is exposed to unsuitable conditions.

Issue: How effective are quality assurance and oversight systems?

Importance: The nature of the disease is such that science-based quality assurance programs that identify critical management phases (e.g., inoculum minimization, maintenance of low thresholds of infection, distribution of symptomless fruit, and fruit disinfestation at the pack-house) require participation by most producers and distributors in a given area. Effective oversight by regulatory bodies will depend on tractable compliance and monitoring systems similar to the Phytosanitary Hazard Analysis and Critical Control Point Systems (PHACCP) programs described elsewhere (APHIS, 2003).

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Figure 1. Life-cycle of *Xanthomonas axonopodis* pv. *citri*, the pathogen causing citrus canker disease

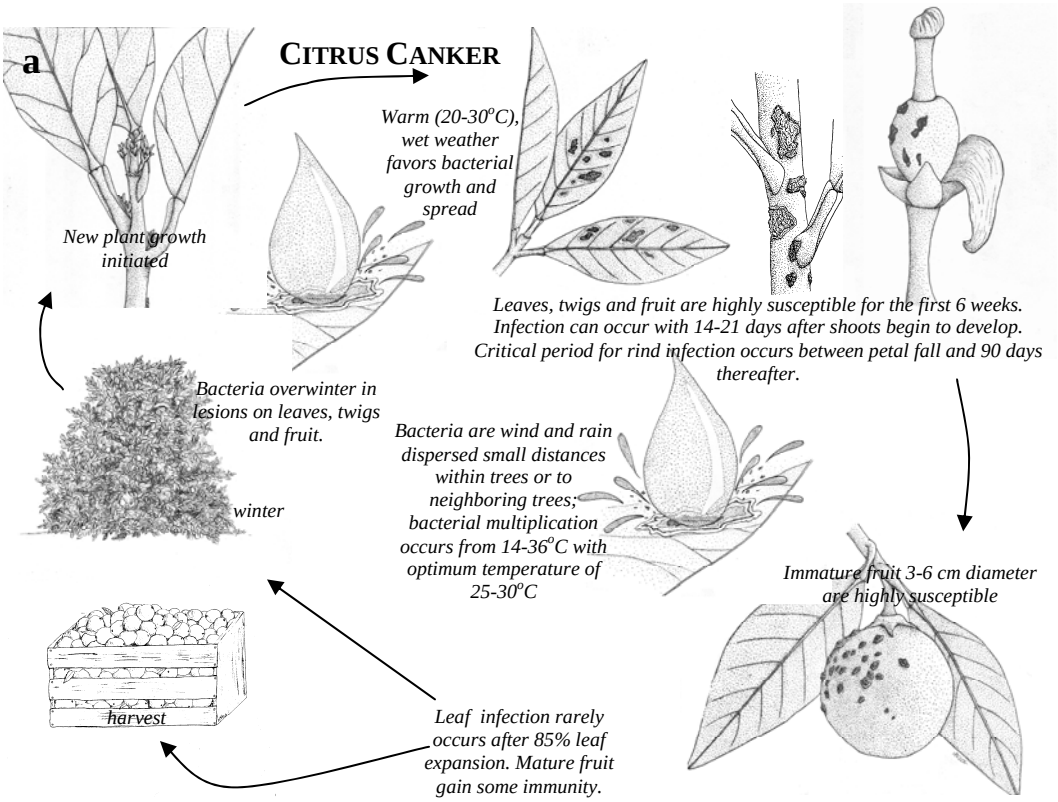


Figure 2. Accumulated favorable events based upon hourly wind and rain data (left) and proportion of citrus grown by county (right). Proportion calculated by dividing total citrus acres per county by total acres per county. The reporting weather stations used in citrus canker spread event analysis are represented by red dots (Borchert et al., 2007).

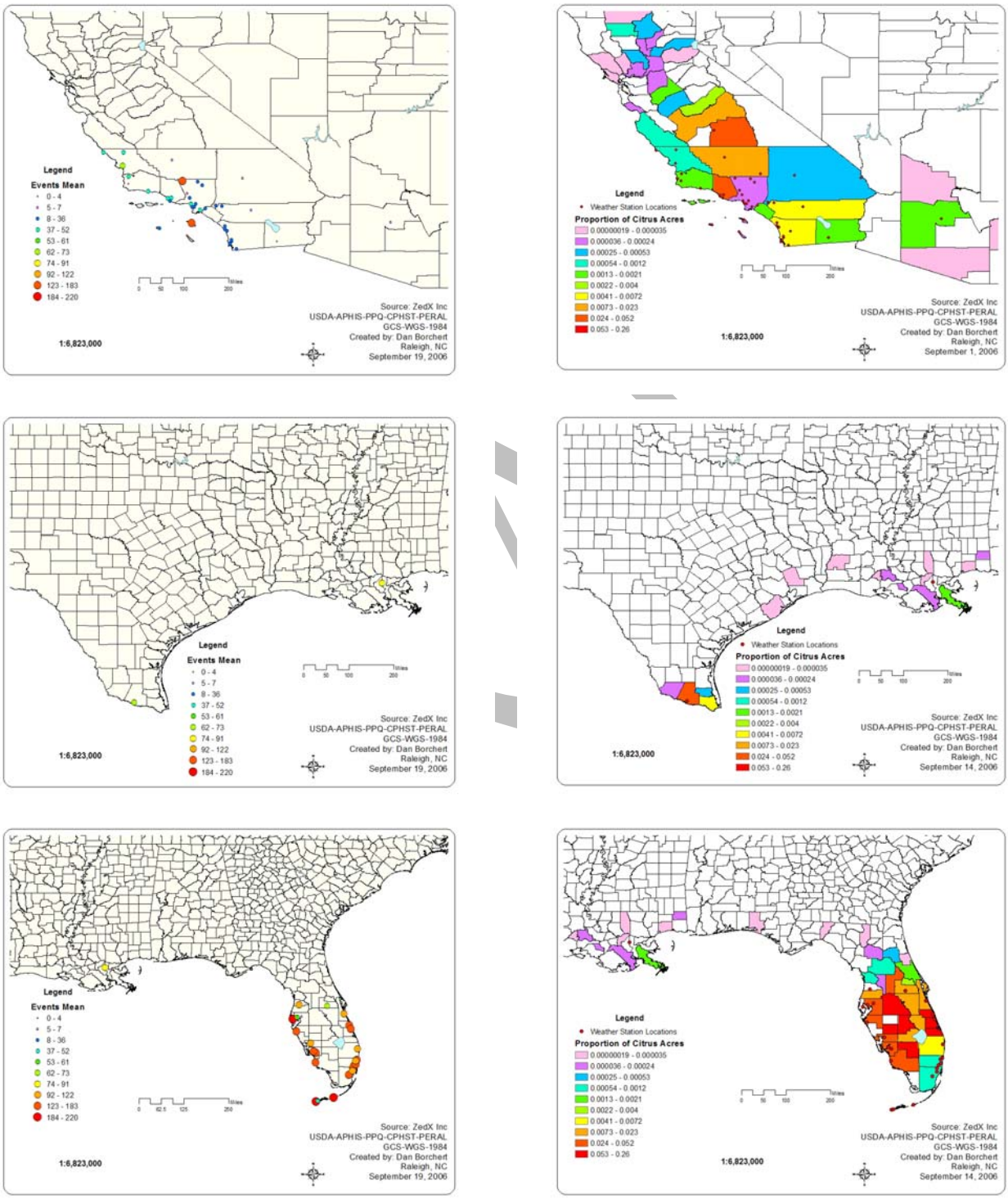
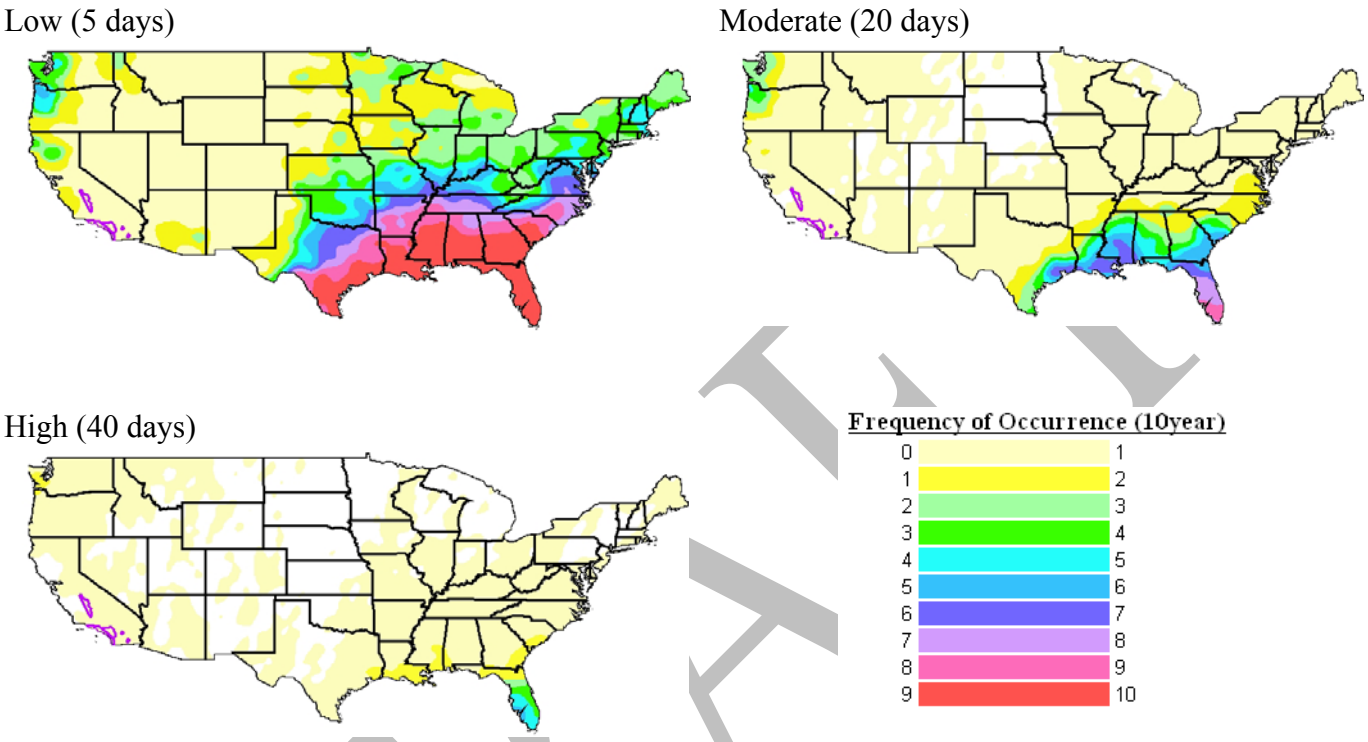


Figure 3. Forecast potential for citrus canker disease in The United States. The NAPPFAST Xac infection model shows the frequency of years having a specific number of days favorable for infection based on both moisture (wetness and rain) and temperature. Frequency of years favorable for Xac infection at three thresholds, low (≥5 days), medium (≥20 days) and high (≥40 days). Pink areas in California represent primary citrus growing regions (Borchert et al., 2007).



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