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New Pest Response Guidelines

Citrus Greening Disease

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Citrus Greening Disease

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Cover Images

Asian citrus psyllid (*Diaphorina citri*) adult courtesy of David Hall, USDA–ARS, Ft. Pierce, FL

Symptoms of citrus greening on lime (*Citrus aurantifolia*) courtesy of Xiaoan Sun, Florida Department of Agriculture and Consumer Services, Division of Plant Industry

Citrus Greening Disease

Contents

1

Citrus Greening Disease

Chapter 1

Introduction

Contents

Introduction

Use *New Pest Response Guidelines: Citrus Greening Disease* as a guide when designing a program to detect, diagnose, contain and control, or eradicate citrus greening disease (CG). Personnel within state departments of agriculture, and others concerned with developing local survey or control programs for CG, should find the *Guidelines* useful.

United States Department of Agriculture (USDA)–Animal and Plant Health Inspection Service (APHIS)–Plant Protection and Quarantine (PPQ) developed the *Guidelines* through discussion, consultation, or agreement with staff at USDA–Agricultural Research Service (ARS), universities, industries, and state departments of agriculture.

The *Guidelines* will be updated as new information becomes available. Specific emergency programs should be based on information available at the time of the incident.

Recent Updates

Significant changes to this document as a result of new information will be provided in this section. Updates to the current version include the following:

- Citrus greening disease is the name currently approved for use in USDA documents, replacing the name huanglongbing disease of citrus. **See Classification on page 2-1** for related information.
- Seed of citrus and citrus relatives are regulated due to the potential for seed transmission of citrus greening (USDA, January 11, 2008). **See Regulated Articles on page 5-5** for related information.
- The diagnostic laboratory with proper authorizations to do final confirmatory testing for *Candidatus* Liberibacter is the USDA–APHIS–PPQ–Molecular Diagnostics Laboratory (MDL) in

Beltsville, MD. MDL is authorized by APHIS to receive suspect domestic select agent plant pathogens under APHIS permit number 65253. **See Identification on page 4-1** for related information.

Status

Citrus greening disease is one of the most serious insect-vectored pathogens of citrus. In August, 2005, CG was detected and confirmed in south Miami-Dade County, Florida. However, a comprehensive delimiting survey for CG was **not** conducted in Florida in 2006. Other citrus growing states conducted general detection surveys in 2006, and no disease was detected.

By mid-2006, the Asian citrus psyllid was widespread throughout the citrus growing areas in Florida and the lower Rio Grande Valley in Texas. The Asian citrus psyllid was also detected in Hawaii in 2006. The African citrus psyllid has **not** been detected in surveys.

Management

See the Web site *USDA–APHIS–PPQ–Citrus Health Response Program (CHRP)* to learn more about the minimum standards for citrus health in Florida.

Address http://www.aphis.usda.gov/ppq/pdmp/citrushealth/

The goals of CHRP are as follows:

- Sustain the U.S. citrus industry,
- Maintain the grower's continued access to export markets, and
- Safeguard the other citrus growing states against a variety of citrus diseases and pests.

This is a collaborative effort involving growers, Federal and State regulatory personnel and researchers.

CHRP provides guidelines for nursery stock product compliance and fruit inspection, treatment, and certification. CHRP will also identify minimum standards, where available, for implementing appropriate survey, diagnostic, and mitigation measures to reduce the proliferation and spread of citrus canker, citrus greening, and other diseases of regulatory significance.

Prevention

Federal and state regulatory officers must conduct inspections and apply prescribed measures to ensure that the disease or pathogen does **not** spread within or between properties. Federal and state regulatory officers conducting

inspections should follow proper sanitation guidelines to prevent spreading contaminated plant material or tools to other facilities before entering and upon leaving each property. For information concerning sanitation, see **Disinfecting Equipment on page F-1**.

Disclaimers and Document Comprehension

This document provides a foundation, based on available literature, to assist further work. This document is **not** intended to be complete and exhaustive. Some key articles were **not** available at the time of writing, and **not** all specialists and members of the research community were consulted for their advice. For the most current information on this pest, consult with local agricultural experts, including personnel from USDA, Cooperative Extension Service, and state departments of agriculture. Conduct your own literature search and review Web sites frequently for updated information.

Endorsement

References to commercial suppliers or products should **not** be construed as an endorsement of the company or product by USDA.

Safety

The safety of the public as well as the program personnel is a priority consideration in preprogram planning and training, and throughout program operations. Safety officers and supervisors must enforce on-the-job safety procedures.

Support for Program Decision Making

The USDA–APHIS–PPQ–Center for Plant Health, Science and Technology (CPHST) provides technical support, in consultation with other scientists, to emergency pest response program directors concerning risk assessments, survey methods, control strategies, and other aspects of pest response programs. PPQ managers consult with state departments of agriculture when developing guidelines and policy for pest response programs.

2

Citrus Greening Disease

Chapter 2

Pest Information

Contents

Classification

Two species of fastidious phloem-limited bacteria—*Candidatus* Liberibacter africanus[1] and *Candidatus* Liberibacter asiaticus—are thought to be the causal organisms of citrus greening disease (CG). These fastidious phloem-limited bacteria have **not** yet been cultured on artificial media and are able to survive only within the phloem vascular system of a plant.

The International Organization of Citrus Virologists adopted huanglongbing as the official name of the disease caused by Liberibacter species in citrus (Lee and Garnsey, 1996). Citrus greening is the term approved for use in USDA documents. Use **Table 2-1 on page 2-2** as an aid to classification of the two species of bacteria that are thought to cause CG.

1 *Candidatus* is the name that plant pathologists use for bacterial species that have **not** yet been successfully cultured. By convention, the actual genus and species names are **not** italicized when the name *Candidatus* is used. Plant pathologists will assign a new name once the bacteria have been cultured and taxonomically described (Halbert and Manjunath, 2004).

TABLE 2-1 Nomenclature of *Candidatus* Liberibacter africanus Garnier, *Candidatus* Liberibacter asiaticus Garnier, and citrus greening disease

Domain	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhizobiales
Family	Rhizobiaceae
Scientific names	*Candidatus* Liberibacter africanus Garnier *Candidatus* Liberibacter americanus Texeira *Candidatus* Liberibacter asiaticus Garnier
Synonyms	*Candidatus* Liberobacter africanus Garnier *Candidatus* Liberibacter americanus Texiera *Candidatus* Liberobacter asiaticus Garnier
Official common name (IOCV, 1996)	huanglongbing (yellow shoot or literally "yellow dragon" in Chinese)
Additional common name (USDA)	citrus greening
Additional common names	citrus greening, CG, HLB, likubin, decline (in Taiwan), leaf mottling (in Philippines), citrus dieback (in India), vein phloem degeneration (in Indonesia), yellow branch or blotchy-mottle (in South Africa)

History

The following two bacterial species were listed as select agents under the Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331):

- *Candidatus* Liberibacter asiaticus Garnier—also known as the Asian form and abbreviated *Ca*. L. asiaticus, and
- *Candidatus* Liberibacter africanus Garnier—also known as the African form and abbreviated *Ca*. L. africanus.

Citrus greening is vectored by the following two species of psyllids:

- Asian citrus psyllid (*Diaphorina citri* Kuwayama), and
- African citrus psyllid (*Trioza erytreae* [del Guercio]) (Aubert, 1987).

A third bacterial species—*Candidatus* Liberibacter americanus—was recently described from Sao Paulo, Brazil (Texiera *et al.,* 2005, Coletta-Filho *et al.,* 2005). This species is not currently listed as a select agent. *Candidatus* Liberibacter americanus appears to be transmitted by the Asian citrus psyllid, or by

grafting (Coletta-Filho *et al.,* 2005). The Asian citrus psyllid is common in Brazil. Research is underway to more fully characterize *Candidatus* Liberibacter americanus.

A review by da Graça and Korsten (2004) provided useful information on CG's history. Farmers in southern China noted a citrus disease, which they called huanglongbing ("yellow dragon"), in the late 1800s. In the early 1920s, similar diseases were reported in the Philippines (mottle leaf), India (dieback), and Taiwan (likubin). A citrus disease, called yellow branch or greening, was noted in South Africa in the late 1920s, and a malady known as citrus phloem degeneration, was reported from Indonesia in the 1940s. The disease has subsequently been found in numerous other citrus producing countries in Africa and Asia.

It was not until the 1960s that the link among the various diseases was established. The disease was initially believed to be caused by drainage problems, while later reports suggested that a mineral deficiency was the cause. Likubin, in Taiwan, was reported to be associated with a nematode problem.

Successful graft transmission of the disorder in the 1940s and insect transmission in the 1960s led to speculation that the causal agent was a virus. In 1970, mycoplasma-like organisms (or phytoplasmas) were putatively observed in the sieve tubes of CG-infected orange leaves. More recent molecular studies have established that the causal organisms are actually true bacteria (da Graça 1991). In 1996, the International Organization of Citrus Virologists officially adopted huanglongbing as the name to describe the disease caused by Liberibacter species in citrus.

The disease has subsequently been found in numerous other citrus producing countries in Africa and Asia. The first report of the disease in the Western Hemisphere came from Brazil in 2004; confirmation of the disease in Florida was announced in a press release on September 2, 2005 (USDA, 2005).

Damage to Hosts

Within the citrus plant, the bacteria are limited to the phloem. This condition leads to the following symptoms:

- Shoot color yellow,
- Leaves with characteristic blotchy mottling,
- Normally green tissue turns yellow (chlorosis),
- Total foliage reduced, and
- Leaf tips dieback (Bové and Garnier, 2002).

Citrus fruits infected with CG have the following characteristics:

- Shape lopsided,
- Size small,
- Color remaining green with seeds aborted (Bové and Garnier, 2002), and
- Taste sour.

The three forms of bacteria produce similar symptoms in citrus. Bacterial strain differences affecting symptom severity have been noted in both the African and Asian forms.

Candidatus Liberibacter africanus Garnier

The African form of the pathogen is heat sensitive, with symptoms produced under relatively cool conditions (20—24° C optimum) (Garnier and Bové, 1993). Extended periods of high temperatures suppress symptom development but do not suppress infection of citrus.

Candidatus Liberibacter americanus Texeira

Little is currently known about the temperature requirements for the American form of the pathogen.

Candidatus Liberibacter asiaticus Garnier

The Asian form usually produces a more severe disease reaction than the African form. This form is also heat tolerant, producing symptoms under cool to relatively warm conditions (up to 32° C) (Bové *et al.*, 1974).

Transmission and Bacterial Development

The disease is graft- and vector-transmissible (Catling, 1970; Catling, 1973; Ghosh *et al.*,1977; Garnier and Bové, 1983; Garnier *et al.*, 1984b; Garnier *et al.*, 1984a; da Graça, 1991; Swai *et al.*, 1992; Kohno *et al.*, 2001).

The bacteria reproduce in the hemolymph and salivary glands of the insects after they feed on infected plants (Catling, 1970; Garnier *et al.*, 1984a; Aubert, 1987; Rae *et al.*, 1997; Subandiyah *et al.*, 2000; Bové and Garnier, 2002). Once

the citrus psyllids acquire the bacteria, they transmit it to new hosts for the remainder of their life cycle in a persistent, propagative manner (Garnier *et al.*, 1984a; da Graça, 1991; Bové and Garnier, 2002). It is **not** known if the bacteria are transmitted transovarially to subsequent generations, but the nymphs and adults can acquire the bacteria in less than one day (Aubert, 1987).

Incubation periods in the vector range from as little as one day (*Ca*. L. africanus) to as long as 12 days (*Ca*. L. asiaticus). Adult insects are then capable of transmitting the bacteria for the remainder of their lives (Aubert, 1987). The 2nd through 5th instar nymphs can also acquire and, under experimental conditions, the 4th and 5th instars can transmit the bacteria.

Only adult psyllids are known to transmit the bacteria under natural conditions.

Economic Impact and Host Range

The host range of the bacteria under natural conditions appears to be restricted to rutaceous plants (members of the plant family Rutaceae), although dodder, periwinkle (Tirtawidjaja, 1981), and tobacco (Garnier and Bové, 1993) have been infected under experimental conditions. CG severely affects sweet orange, mandarin and tangelo trees, but susceptibility among other *Citrus* species varies (Garnier *et al.,* 1984a; Bové and Garnier, 2002). Mexican lime (*Citrus aurantifolia*) is less susceptible than sweet orange and mandarin even though it is a preferred host of the Asian citrus psyllid (Garnier *et al.*, 1984a; Bové and Garnier, 2002).

Several wild and ornamental rutaceous species are hosts of the psyllid vectors, such as orange jasmine (*Murraya paniculata)* and curry leaf tree (*Murraya keonegii);* however, the literature is inconclusive as to the status of *Murraya* spp. as hosts for the CG pathogens (Halbert and Manjunath, 2004).

By the early 1990s, CG had become widespread throughout the citrus growing regions of Asia and the southern and eastern parts of Africa. Infection with CG resulted in the estimated loss of over 60 million citrus trees by the early 1990s. The following examples serve to illustrate the seriousness of both the Asian and African forms of this disease.

Four million citrus trees were eradicated on the island of Bali from 1986 to 1988. These trees were replaced with mandarins in 1991. By 1993, 40% of the replacements were infected with CG, increasing to a 90% infection rate by 1996.

The area planted to citrus in the Philippines was reduced by over 60% between 1961 and 1970 due to CG, with a loss of over one million trees recorded in one province in 1971. Many trees in Thailand die or go out of production within 5-6 years after planting.

An estimated 4 million of the 11 million citrus trees planted in South Africa were infected with CG by the mid 1970s. By this time, three major production areas, representing 20% of the citrus industry, had been eliminated due to the disease.

Plant Hosts

Rutaceous plants are the natural hosts of liberibacter species, with all species and cultivars of citrus susceptible to infection (Garnier *et al.,* 1984; Bove and Garnier, 2002). Sweet oranges (*Citrus sinensis*), mandarins (*C. reticulata*), and mandarin hybrids are the most severely affected. Grapefruit (*C. x paradisi*), sour oranges (*C. aurantium*), and lemons (*C. limon*) are moderately affected. Mexican (Key) lime (*C. aurantifolia*), pummelo (*C. maxima*) and trifoliate orange (*Poncirus trifoliata* Raf.), including its hybrids, are the most tolerant. Kumquat (*Fortunella spp.*) is also a host.

A number of other rutaceous plants have been observed to be hosts of both the bacteria and the citrus psyllid, either experimentally or naturally: *Severinia buxifolia*, *Balsamocitrus dawei*, *C. grandis*, *C. hystrix, C. jambhiri*, *Citrus* x *nobilis*, *Clausena indica, Cl. lansium, Microcitrus australisica*, *Triphasia trifolia, Atalantia missionis*, *Severinia buxifolia*, *Limonia acidissima* (= *Feronia limonia*), and *Swinglea glutinosa* (Hung *et al.,* 2000; Hung *et al.,* 2001; Halbert and Manjunath, 2004).

Several wild and ornamental rutaceous species are hosts of the psyllid vectors, such as orange jasmine (*Murraya paniculata*), curry leaf plant (*Murraya keonegii*), jackfruit (*Artocarpus heterophyllus),* and cape chestnut (*Calodendrum capense* Thunb). *Murraya* spp. are hosts for the vector and the pathogen (Deng, *et al*., 2007). Specimens of *Calodendrum capense* were found that were infected with a distinct subspecies of *Candidatus* Liberibacter africanus.

Some of these ornamentals may be used in warm climate areas of the United States. Orange jasmine is a preferred host of the Asian citrus psyllid, and has likely aided the spread of this insect throughout the citrus growing areas of Florida and Texas. The plant *Toddalia lanceolata* (=*Vepris undulata*), considered to be one of the original hosts of the African citrus psyllid, is also a host of the African form of CG.

Dodder has been used to experimentally transmit both *Candidatus* Liberibacter africanus and *Ca*. Liberibacter asiaticus. *Cuscuta reflexa* has been used for citrus-to-citrus transmission, and *C. campestris* has been used to transmit the pathogens to periwinkle (*Catharanthus roseus* Don.) with marked foliar yellowing. *Candidatus* Liberibacter asiaticus has also been transmitted to tobacco (*Nicotiana tabacum* var. *xanthi* NC L.) via dodder. Dodder itself appears to be a host to the bacteria, but in the epidemiology of the disease, dodder as a host and potential vector of the pathogen is **not** likely to be significant.

See **Regulatory Procedures on page 5-1** for a list of regulated hosts.

Geographic Distribution

Pathogens causing CG have been reported from the following countries in Africa, Asia and South America: Bangladesh, Bhutan, Brazil, Burundi, Cambodia, Cameroon, Central African Republic, China, Comoros, Ethiopia, Hong Kong, India, Indonesia, Japan, Kenya, Laos, Madagascar, Malawi, Malaysia, Mauritius, Myanmar, Nepal, Pakistan, Papua New Guinea, Philippines, Reunion, Rwanda, Saudi Arabia, Somalia, South Africa, Sri Lanka, Swaziland, Taiwan, Tanzania, Thailand, Vietnam, Yemen, and Zimbabwe.

To date, CG pathogens have **not** been reported from citrus-producing regions of Australia, Mexico, countries in Central America or the Mediterranean.

Development of Citrus Greening Disease

Citrus greening is vectored by the following two species:

- African citrus psyllid (*Trioza erytreae* (del Guercio) (Aubert, 1987)), and
- Asian citrus psyllid (*Diaphorina citri* Kuwayama).

The bacteria can also be transmitted in orchards or nurseries by grafting, and experimentally by several species of dodder (*Cuscuta* spp.) (Halbert and Majunath, 2004). Natural transmission of *Ca.* L. africanus is facilitated by both the African and Asian citrus psyllids.

Ca. L. asiaticus and *Ca.* L. americanus are vectored by the Asian citrus psyllid (Teixeira *et al.*, 2005). However, each psyllid species has been demonstrated to transmit either the African or the Asian form of the bacteria experimentally.

Candidatus Liberibacter Species

The bacteria have **not** yet been successfully cultured outside of the phloem of citrus plants or the psyllid vectors (Garnier *et al.*, 1984a; da Graça, 1991; Bové and Garnier, 2002). *Candidatus* Liberibacter africanus, present in South Africa, Kenya, Ethiopia, Madagascar and Yemen, is heat sensitive and unable to cause symptoms at temperatures above 25-30°C, whereas *Ca.* L. asiaticus, which occurs throughout much of Asia, India, and Indonesia, is heat-tolerant and able to cause symptoms at temperatures above 30°C (Garnier *et al.*, 1984a; da Graça, 1991; Bové and Garnier, 2002).

The liberibacters inhabit the nutrient-rich phloem. Other similar organisms cause more than twenty diseases of plants, including papaya bunchy top, watermelon yellow vine, and strawberry marginal necrosis (Kiritani and Su, 1999; Bové and Garnier, 2002). None of the organisms have been cultured (Bové and Garnier, 2002).

When the 16s ribosomal DNA of *Candidatus* L. africanus was amplified, the DNA was most similar to the bacteria of the α subdivision of *Proteobacteria*, which includes plant and human pathogens such as *Agrobacterium tumefaciens*, *Bradyrhizobium* spp., and *Brucella abortus* (Jagoueix *et al.,* 1994; Bové and Garnier, 2002).

Psyllid Vectors

The African and Asian citrus psyllids are the only known insect vectors of the CG pathogens. The Asian citrus psyllid is now widespread throughout the citrus growing areas of Florida (Halbert *et al.,* 1998, Tsai *et al.,* 2002) and the lower Rio Grande Valley of Texas (French *et al.,* 2001).

The host range of Asian citrus psyllid is restricted to citrus and closely related Rutaceae (Aubert, 1987, Halbert and Manjunath, 2004). The preferred host is *Murraya paniculata*, an ornamental rutaceous plant called orange jasmine (also known as orange jessamine and commonly in the Florida nursery trade as Lakeview, a cultivar) found throughout the citrus belt in its native range (Kohno *et al.,* 2001) and often planted in the southeastern United States as an ornamental hedge plant. The Asian citrus psyllid recently spread to all citrus growing areas in Florida with the host plant (Halbert and Majunath, 2004). However, orange jasmine is **not** currently considered a host for the liberibacters (Hung *et al.,* 2000).

Adult citrus psyllids are small (3 to 4 mm) with mottled brown wings. Adults are active, jumping insects. Eggs are bright yellow and deposited on newly emerging citrus tissue. Nymphs are green or dull orange, and feed on leaves and stems where they are difficult to see. Asian citrus psyllids are most likely to be found on new shoots, and population increase occurs during periods of active plant growth (Aubert, 1987).

The Asian citrus psyllid has a light brown head, while African citrus psyllid adults have a black head. When disturbed, the adult psyllids move or jump quickly, occasionally flying short distances. Nymphs of the Asian citrus psyllid are light yellow to dark brown, with large, well-developed wing pads. Nymphs of the African citrus psyllid vary in color from yellow to olive-green to dark gray, with marginal fringes of white, waxy filaments and small wing pads. Find additional information on psyllid identification in **Identification of Psyllids on page D-1**.

High population densities of Asian citrus psyllid stunt and twist young shoots, causing a rosette appearance. Leaves become curled, but do **not** contain the pit galls typical of the African citrus psyllid. High population densities of African citrus psyllid severely distort leaves, which are stunted and contain typical pit galls.

Vector Pathogen Interaction

Psyllid numbers increase in late winter and spring when the citrus trees are flushing (new foliage growth) and adults may fly for short distances. Cool, moist conditions favor increased populations of African citrus psyllid, while

the Asian citrus psyllid prefers a warmer, drier environment. High psyllid population densities are often found in citrus nurseries, since the young trees are maintained in a state of almost constant growth. Active growth on alternate plant hosts support psyllid populations when citrus flush is not available.

The interaction between the vector and the pathogen is poorly understood. Acquisition times of between 30 minutes and 24 hours have been reported (Aubert, 1987). The pathogen also multiplies in the vector. Adults and fourth and fifth instar Asian citrus psyllid nymphs can transmit *Ca*. L. asiaticus after 8-12 days, with a shorter latent period of 1 day reported for African psyllids.

Following a 1-21 day incubation period (average 7-12 days), the psyllids are able to transmit the bacteria for the rest of their lives (average lifespan of 60 days). A 2nd or 3rd instar could acquire the pathogen and become increasingly infective in the 4th or 5th instar (Hung *et al.,* 2004). Nymphs, however, would remain on the CG-infected host material from which it acquired the *Ca*. L. asiaticus pathogen and pose little risk of spreading the disease to new material until reaching the adult stage.

Recent experiments by Hung *et al.,* (2004) showed the pathogen is **not** transmitted transovarially (from adult to egg).

Nymphs reportedly do **not** transmit the pathogen in the field, although they are able to acquire it when feeding on infected plants and later transmit it as adults. Late-instar nymphs are, however, able to transmit the bacteria when moved from infected to healthy plants under experimental conditions. Most adult insect movement is within the host plant or to nearby plants, but the psyllids are capable of flying considerable distances (1.5 miles has been documented) in search of suitable hosts.

Not all plants the psyllids feed on are hosts of the bacteria. For a complete list of psyllid hosts, see Halbert and Manjunath (2004).

3

Citrus Greening Disease

Chapter 3

Survey Procedures

Contents

Introduction

Plant regulatory officials conduct detection, delimiting, and monitoring surveys. Detection surveys are performed to ascertain the presence or absence of a pest in an area where it is not known to occur. Delimiting surveys are performed to define the extent of an infestation. Monitoring surveys are performed to determine the success of control or mitigation activities conducted against a pest.

Use this chapter as a guide to conducting a survey for citrus greening disease (CG) and its vectors.

Precautions

Take the following precautions before starting a survey.

Pesticide Applications

Before starting a survey, always determine if there have been recent pesticide applications that would make it unsafe to inspect the citrus. Check with property owners or managers for this information. Look for posted signs indicating recent pesticide applications, particularly in commercial groves.

Quarantines

Determine if any quarantines are in effect for the area being surveyed, such as for root weevils or citrus canker. Comply with all quarantine requirements.

Private Property

Obtain permission from the landowner before entering a new property. See **Regulatory Procedures on page 5-1** for pertinent information.

Sanitation

When visiting nurseries to conduct surveys or collect samples, regulatory officials must take strict measures to prevent contamination by plant pathogens between properties during inspections. Viroids—small pieces of naked RNA similar to a plant virus but lacking the protein coat—are extremely difficult to remove from tools. Most disinfectants, as well as heat, fail to inactivate viroids.

When collecting samples, disinfect pruning shears between cuts and prior to use on a new property, to avoid spreading citrus exocortis or other citrus viroids. These citrus pathogens can be carried on the cutting surfaces of pruning shears, knives, and other implements used for cutting and pruning operations. Making a cut on an infected tree is sufficient to contaminate the cutting tool; subsequent cuts on other trees will introduce the viroid and infect the tree.

See **Disinfecting Equipment on page F-1** for instructions on how to use a 5% solution of common household bleach (sodium hypochlorite) to inactive citrus viroids.

Survey Strategies

Conduct a detection survey to determine if CG is present or absent in a defined area. After a new U.S. detection, or when a detection in a new area is confirmed, conduct a delimiting survey to define the geographic location of the disease. Conduct a monitoring survey if you have applied a control procedure and need to measure its effectiveness.

Additional types of surveys may be appropriate. See **Survey for Satellite Infestations on page 3-10**, **Vector Survey for Predicting Future Outbreaks on page 3-10**, and **Surveys of Other Hosts on page 3-11**.

Survey task forces should consist of an experienced survey specialist or plant pathologist familiar with the disease symptoms and personnel responsible for sample collection and proper recording the data and GPS coordinates.

Detection Survey

Perform a detection survey to ascertain the presence or absence of a pest in an area where it is not known to occur.

A detection survey can be broad in scope—as when assessing the presence of the disease over large distances—or it may be restricted to determining if a specific pest is present in a focused area.

Statistically, a detection survey is **not** a valid tool to claim that a pest does **not** exist in an area, even if results are negative. Use negative results to provide clues to mode of dispersal, temporal occurrence, or the effects of local industry practices. Compilation of results are also important particularly when considered with results from similar that are topographically, spatially, or geographically similar.

Survey for CG over a broad area on a regular basis, using various techniques. General survey strategy may be focused on finding disease symptoms, vectors, or both, depending on the target location. Use **Table 3-1 on page 3-4** to determine the type of detection survey strategy to use.

TABLE 3-1 Decision table to determine detection survey strategies focused on finding disease symptoms or vectors

If you want to detect the following:	And:	Then conduct this type of detection survey:	And follow this procedure:
CG disease symptoms	One of the vectors is known to occur in the State	**Symptom Survey on page 3-5**	1. Train surveyors to recognize the symptoms of CG infection on various hosts. 2. Direct trained surveyors to look for those symptoms.
CG vectors	The vectors are known to occur in the survey area	**Vector Survey on page 3-5**	1. Use aspirators, a sweepnet, or detection traps to collect psyllid specimens. 2. Use molecular diagnostic techniques to analyze psyllid specimens for the presence of the CG pathogen.
	The vectors are **not** known to occur in the survey area	**Vector Survey for CG on page 3-5**	Use the following tools: ◆ Visual inspection (see **Visual Inspection for Detection Survey on page 3-11**), ◆ Sweepnets (see **Sweepnet on page 3-13**), ◆ Trapping (see **Detection Traps on page 3-13**), and ◆ Tap Sampling (see **Tap Sampling on page 3-13**).
CG disease symptoms and vectors	**Neither** CG or the vectors have been found in the State	**Sentinel Survey on page 3-5**	1. Train surveyors to do the following: ➸ Recognize the symptoms of CG infection and its vectors (see **Similar Symptoms on page C-6**), and ➸ Set traps for vectors. 2. Select a sentinel tree (of a susceptible species) in an area where CG and the vectors have not been found. 3. Direct trained surveyors to set traps in sentinel trees, then inspect the following: ➸ Sentinel trees for signs of CG infection, and ➸ Traps every 30—90 days for trapped vectors.
	The area is residential and at high risk	**Targeted Survey on page 3-6**	1. Request a query from PPQ–National Identification Services–Pest Interception Database. 2. Search for *Citrus* spp. plants seized in passenger baggage for any pest. 3. Compare CG world-wide distribution with results from previous step (2), then use United States census data to identify zip codes with a higher number of residents that may have originated in those countries. 4. Make contact with residents before inspecting trees on their property.

Detecting Disease Symptoms

Symptom Survey—In states where one of the CG insect vectors is known to occur, follow these steps to conduct a symptom survey for the disease:

1. Train surveyors to recognize the symptoms of CG infection on various hosts (see **Visual Inspection for Detection Survey on page 3-11**), and
2. Direct trained surveyors to look for those symptoms.

Detecting Vectors

Vector Survey—If the psyllid vectors are **not** known to occur in the survey area, use the following tools to detect them during a survey:

- Visual inspection (see **Visual Inspection for Detection Survey on page 3-11**),
- Sweepnets (see **Sweepnet on page 3-13**),
- Detection Traps (see **Detection Traps on page 3-13**), and
- Tap Sampling (see **Tap Sampling on page 3-13**).

Winged adults are easier to see than nymphs because they appear flattened on a host leaf surface similar to scale insects. Flush growth on host plants is the most likely place to find adults and nymphs.

Conduct a vector survey in citrus nurseries that sell orange jasmine (*Murraya paniculata*). Orange jasmine is the preferred host of the Asian citrus psyllid (*Diaphorina citri*). Do **not** analyze orange jasmine for the presence of the CG pathogen.

Vector Survey for CG—If the psyllid vectors are known to occur in an area, follow this sequence to detect CG in vectors:

1. Use aspirators, sweepnets, or detection traps to collect psyllid specimens.
2. Use molecular diagnostic techniques to analyze psyllid specimens for the presence of the CG pathogen.
3. If vectors are confirmed positive for CG in a state or county where the disease has **not** been confirmed in plant tissue, continue to survey the area for plant disease symptoms. Take both symptomatic and non-symptomatic leaf samples from the tree where the positive psyllids were collected. Also look for symptoms on other host trees in the residential area or grove surrounding the site.

Detecting Disease and Vectors

Sentinel Survey—Sentinel trees are specific trees selected to conduct surveys on a repeated basis (every month or several times per year). In states where CG and the vectors have **not** been found, survey specific designated trees on a continuing basis. Conduct a sentinel survey in residential or commercial groves. Follow this sequence:

1. Train surveyors to do the following:

A. Recognize the symptoms of CG infection and its vectors (see **Similar Symptoms on page C-6**), and

B. Set detection traps for vectors.

2. Select a sentinel tree in an area where CG and the vectors have **not** been found. Select a susceptible species as a sentinel tree. The most susceptible CG hosts are orange, mandarin, tangelo, and tangerine. The most susceptible psyllid hosts are *Murraya* spp. and *Citrus* spp.
3. Direct trained surveyors to set detection traps in sentinel trees, then inspect the following:
   - ❖ Sentinel trees for signs of CG infection, and
   - ❖ Traps every 30—90 days for trapped vectors[1].

Targeted Survey—Consult PPQ–National Identification Services or a PPQ Identifier to conduct a query from the Pest Interception Database (commonly referred to as Pest ID) system. Target the survey in residential neighborhoods that are at high risk. Using Pest ID, search for *Citrus* spp. plants seized in passenger baggage for any pest. Results will indicate source countries for host plants carried by passengers. Couple the CG world-wide distribution with this list and then use United States census data to identify Zip Codes with a higher number of residents that may have originated in those countries. Surveys can be then targeted in higher risk areas.

Targeted surveys are useful in detecting new foci of infection from unauthorized importations of infected plant material. Outreach materials can be distributed door-to-door. Make contact with residents before inspecting trees on their properties.

Delimiting Survey

After a new U.S. detection, or when a detection in a new area is confirmed, conduct a delimiting survey to define the geographic location of the pest.

Surveys after the first U.S. detection should be most intensive around the known positive detection(s) and any discovered through traceback and trace forward investigations. The intensity of survey sampling around the known positives will lessen in concentric circles away from the positive points on a map. The level of sampling in various radii away from the known infestation will depend on recommendations from USDA–CPHST–ARS, and other scientists, and will also depend on resources available. These surveys should include residential, nursery, and commercial groves with the results mapped to develop potential quarantine boundaries.

Because the disease is distributed randomly, all citrus trees—if possible—should be inspected for the presence of CG symptoms. Be aware that symptoms may occur on only one or a few branches, or (in later stages of

1 If psyllids are being trapped for PCR analysis, they should **not** be left in traps for more than 48 hours before analysis.

infection) throughout the canopy of the tree. Foliar symptoms resemble certain mineral deficiency symptoms and those produced by several other citrus diseases.

When in doubt, always collect and submit samples for laboratory analysis. **See Procedure for Sampling and Identification on page 4-4** for more information on laboratory analysis.

The use of a sentinel tree system like that in place for citrus canker in Florida would be an appropriate survey strategy for this disease, but a more intensive stratified survey around known positive detections will help delimit the extent of the disease in that area.

Data collection can be simplified by the use of preprogrammed hand held units that allow ease of data recording with GPS capability. If available, use PPQ–Integrated Survey Information System (ISIS) for gathering and reporting survey information. ISIS is an internal database. Consult the PPQ Pest Survey Specialist in your area for more information.

Collect the following data during surveys:

- Date of collection,
- Sample number from predetermined numbering system,
- Collector's name and agency,
- Full address including county,
- Type of property (residential, nursery, commercial grove, feral or abandoned grove),
- Grower's field identification numbers (if appropriate),
- GPS coordinates of the host plant and property,
- Species and cultivar of host plant,
- Observations of the number of infected trees,
- Presence of vectors,
- General conditions or any other relevant information, and
- Positive or negative results from testing (recorded later).

Recording negative results in surveys is just as important as positive detections, since it helps define an area of infestation. A system of data collection should include an efficient tracking system for suspect samples such that their status is known at various stages and laboratories in the confirmation process.

Rapid Delimiting Survey—A rapid delimiting survey is one which uses concentric annuli in circular transects. Survey task forces start surveying at one or more known positive host plants in a given location, then conduct inspections in increasing five mile increments along the arcs of concentric annuli.

Depending on the availability of hosts and survey resources, the first 5 mile annulus has 16 equally spaced survey points around the circle. As suspect positive hosts are discovered in the first 5 mile arc, the next survey points will be in a 10 mile annulus, with 32 points, and 15 miles with 64 points (**Figure 3-1 on page 3-8**).

If **no** suspect positive hosts are discovered at a five mile increment, survey crews begin to work back toward the center point to define the delimitation of the infestation. At each sampling point, surveyors will search for the nearest host tree in the immediate area for susceptible hosts (**Figure 3-1 on page 3-8**).

Use the following order of plant preference when sampling:

1. Orange, mandarin, tangelo, and tangerine
2. Pummelo, grapefruit, and sour orange
3. Lemon and lime

Examine trees for the presence of yellow shoots, foliar mottling, zinc pattern deficiency, and yellow veins. **See Symptoms On Citrus on page C-1** to view images of symptoms.

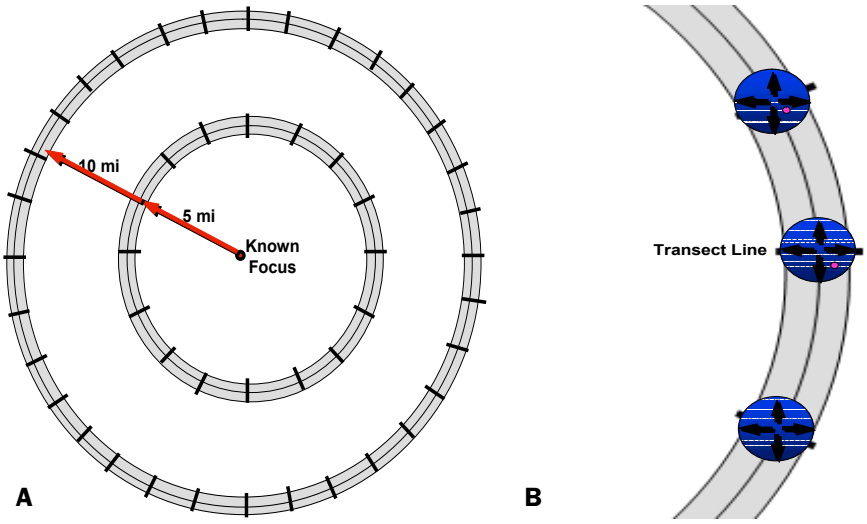

FIGURE 3-1 (A) Sampling points along concentric annuli transects at 5-mile increments away from a known positive host tree (B) Sampling points along an arc transect showing where searching begins to find the nearest host tree for survey

If **no** disease symptoms are found or **no** hosts are found in the sampling point area, search for hosts and symptoms at points adjacent on the same annulus. Depending on the survey crew's instructions, and with **no** evidence of the disease, searches can begin in 1-mile increments back toward the center of the sampling annuli (**Figure 3-2**). At each sampling point, surveyors will search for the nearest host tree in the immediate area for susceptible hosts.

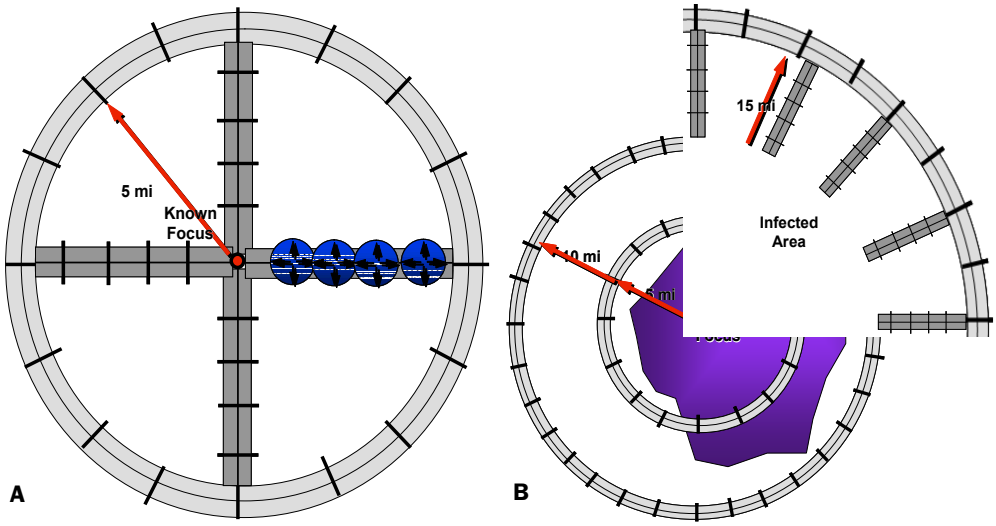

FIGURE 3-2 (A) If disease is not found in the transect sampling point, travel back toward center sampling in one mile increments. (B) If disease is found to extend beyond an annulus in one or two directions, use a partial annulus combined with information from other transects to define the boundaries of the infestation.

Use the following order of plant preference when sampling:

1. Orange, mandarin, tangelo, and tangerine
2. Pummelo, grapefruit, and sour orange
3. Lemon and lime

Examine trees for the presence of yellow shoots, foliar mottling, zinc pattern deficiency, and yellow veins. **See Symptoms On Citrus on page C-1** for images of symptoms.

Further narrowing of infestations boundaries is possible by continuing sampling in different directions along newly defined transects along the annuli. Once suspect samples have been identified and new positive locations are mapped, the points around the original positive trees will more clearly define the extent of the infestation. Refinements will occur with increased sampling.

Depending on the configuration of the survey area, linear transects can be used instead of concentric annuli for conducting a rapid delimiting survey.

Survey for Satellite Infestations

After one or more infestations are delimited, regulatory and control measures may require the removal of exposed hosts around the known infested areas. Further surveys will be necessary to discover satellite infestations or other areas of potential infection. Design a sentinel or other stratified survey to accomplish this (**Figure 3-3**). **See Sentinel Survey on page 3-5** for more information.

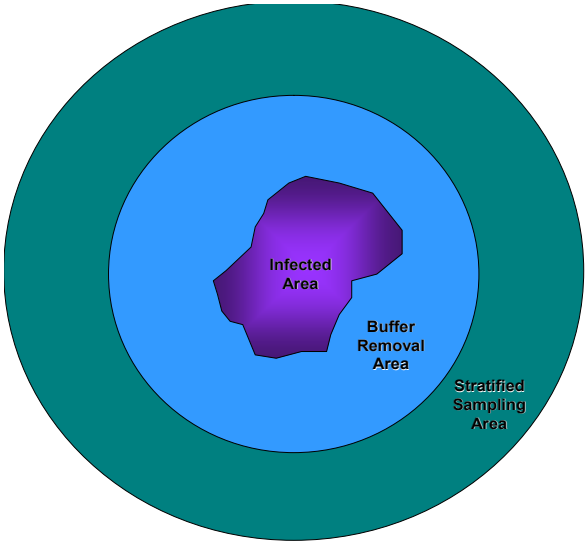

FIGURE 3-3 Stratified survey to detect satellite infestations after delimiting survey

Vector Survey for Predicting Future Outbreaks

Citrus psyllids that are in a known area of CG infestation may be subjected to PCR diagnostics to determine their status as carriers of the pathogen.

See **See Psyllid Samples on page 4-6** and **Identification of Psyllids on page D-1** for methodology when submitting psyllid vectors for identification, and for the testing of psyllid vectors for the presence of disease. Collect psyllids with aspirators, sweep-nets, or detection traps. Collection of psyllids for this purpose should include all appropriate data as plant samples. Conduct vector surveys on the edge of the citrus grove, where psyllid populations might be higher.

Overlay results in map layers with known plant infections and citrus occurrence. Analysis of the maps can help predict the occurrence of CG in subsequent seasons.

Monitoring Survey

Inspections during the same, or subsequent, growing seasons are appropriate in order to examine hosts for symptoms. After any control or eradication procedures are conducted, it is necessary to do follow-up monitoring surveys to assess the success of the program. Also, in areas where control actions have occurred and trees have been removed, monitor for new host plants that may have sprouted from roots remaining in the ground. The length of time for this monitoring should be ascertained in consultation with the Huanglongbing Science Panel. **See Citrus Greening Science Panel on page -ii** for a list of members.

Surveys of Other Hosts

Inspect and sample other ornamental and native hosts in the area for symptoms. These plants may serve as reservoirs for the pathogen. **See Regulatory Procedures on page 5-1** for more information.

Survey Tools

Citrus Greening Disease

Use visual inspection as a tool when surveying for CG.

Visual Inspection for Detection Survey

Limitations—Symptoms alone are **not** diagnostic. Other plant pathogens or cultural conditions (fertility, weather, etc.) can cause similar symptoms. Do **not** conclude that a plant with the symptoms described below is infected with one of the three *Candidatus* Liberibacter species. Assume that the plant is suspect and take samples for further testing.

CG can be difficult to find for the following reasons:

- Symptoms are lacking in its early stages (long latent period);
- Current diagnostic methods fail to detect it;
- Before detection occurs, the pathogen may be vectored over a wide area.

The normal interval between vector inoculation and symptom development ranges from 4—12 months (Hung *et al*., 2000), but the latency period can be longer.

Symptoms on Foliage—The foliar symptoms mentioned in this section are **not** limited to CG. Symptoms of CG can resemble mineral deficiencies [zinc, iron, and manganese (see **Similar Symptoms on page C-6**)] and other diseases (blight, stubborn, and tristeza).

Early foliar symptoms include the following:

- Yellowing of leaves—along the midrib and larger veins—spreading to produce a blotchy, mottled appearance (see **Figure C-7 on page C-3** and **Figure C-9 on page C-4**); and

- Yellow shoot appears on the tree (see **Figure C-6 on page C-2**).

Yellowing of leaves may **not** be noticed until the yellow shoot appears on the tree. The changes are usually confined to one limb or sector of the tree; other limbs bear leaves and fruit that appear healthy. However, if infected at an early age, the yellowing may spread to the entire tree.

Infected sectors of trees exhibit the following symptoms on leaves:

- Leaves small, sparse, upright; and
- Appearance of zinc deficiency symptoms including green veins with chlorotic interveinal areas (see **Figure C-12 on page C-6**).

Leaves with CG have a mottled appearance that differs from nutrition-related mottling. HLP-induced mottling usually crosses leaf veins. Nutrition related mottling usually occurs between or along leaf veins. However, in both cases, leaves may be small and upright.

Symptoms on Fruit—Unlike foliage, symptoms on fruit are much more characteristic of and specific to this disease, although there is some overlap with the symptoms produced by several other citrus diseases.

Symptoms on fruit include the following:

- Fruit is smaller than normal;
- Fruit is usually lopsided (caused by a curved columella):
  - Shaded side remains green
  - Normal coloring develops on unshaded side (see **Figure C-10** and **Figure C-11 on page C-5**)
- Fruits with a somewhat salty, bitter taste (unsalable); in contrast, fruit with similar symptoms caused by other citrus diseases is generally sweeter than normal;
- Seeds generally aborted; and
- Fruit drop is heavy.

Other Symptoms—Twig dieback can occur in severe cases. Heavy leaf abscission and fruit drop, followed by out of season flushing and bloom, can occur on infected trees and branches. Young (1-2 year old) trees may die from the infection. Symptoms of feeding by certain psyllid species may also be used as a possible indicator of their presence. See images of pit-galls caused by African citrus psyllid in **Figure C-4 on page C-1**.

Citrus Psyllids

Use visual inspection, a sweepnet and traps as tools when surveying for citrus psyllids.

Visual Inspection

Inspect the new flush growth of citrus plants for the presence of psyllids. Use an aspirator to collect the psyllids and place them in alcohol.

Sweepnet

Use a sweepnet to collect psyllids in foliage of citrus or other hosts. Preferred citrus hosts or orange jasmine (*Murraya paniculata*) are more likely to yield positive samples if psyllids are present in the area. Take several sweeps against foliage in 15 inch arcs. Choose sites in a variety of geographic areas where hosts occur, especially in urban areas or areas around nurseries. Examine the insects under a dissecting microscope. Submit suspect specimens to an identifier.

Detection Traps

Use yellow or blue sticky cards or suction traps to detect psyllids. Place yellow or blue sticky cards at 0.5 meters in height on or within preferred host plants. Check traps regularly and have an identifier examine them. Suction traps have been used experimentally and may be effective for monitoring pysllids in flight.

Tap Sampling

Use a short piece of PVC pipe or a stick to firmly tap a branch while holding a pan or beating sheet under it. Psyllids or other insects present will fall on the sheet and can be aspirated before taking flight (Hall, *et al*. [in press]).

Traceback and Trace Forward Investigation

Traceback and trace forward investigations help determine priorities for delimiting survey activities after an initial U.S. detection. Traceback investigations attempt to determine the source of infection. Trace forward investigations attempt to define further potential dissemination through means of natural and artificial spread (commercial or private distribution of infected plant material). Once a positive detection is confirmed, investigations are conducted to determine the extent of the infestation or suspect areas in which to conduct further investigations.

Homeowner Properties

For positive detections on homeowner properties, ask the owner of the infected material to determine where it originated (nursery, neighbors, etc.) and where it might have been further distributed.

Nursery Properties

For nursery hosts, compile a list of facilities associated with infected nursery stock from those testing positive for CG. The lists will be distributed by state to the field offices, and are **not** to be shared with individuals outside

USDA–APHIS–PPQ regulatory cooperators. Grower names and field locations on the lists are strictly confidential, and any distribution of lists beyond appropriate regulatory agency contacts is prohibited.

When notifying growers on the list, be sure to identify yourself as a USDA or state regulatory official conducting an investigation of facilities that may have received CG infected material. Speak to the growers or farm managers and obtain proper permission before entering private property.

Several actions need to occur immediately upon confirmation that a citrus nursery sample is positive for CG:

- Check nursery records to obtain names and addresses for all sales during the prior six months. These should be grouped in three sales categories prior to the confirmation of CG:
  - Past one month,
  - Past three months, and
  - Past six months.
- Evaluate the disease situation, including identification and inspection of the budwood source(s) of the diseased tree(s), the location within the nursery, and the disease severity.

See Regulatory Procedures on page 5-1 and **Control on page 6-1** for more information).

Guidelines for Conducting Surveys

Follow these guidelines when designing a program to survey residential areas, commercial groves, or nurseries, for CG or its vectors.

Residential Areas

Important Follow sanitation precautions to avoid spreading plant disease. **See Sanitation on page 3-2** and **Disinfecting Equipment on page F-1** for more information.

Inspect citrus trees on the property for CG symptoms and the presence of citrus psyllids. Examine newer foliage and branches on larger trees, since older foliage is frequently spotted by other pathogens or covered with sooty mold, which can make it difficult to see CG symptoms.

Twigs, Leaves and Petioles

If symptoms are found on a tree, collect samples from those areas of suspect trees that show the best symptoms. Collect green twigs (6—8 inches long) with leaves and petioles attached. Some asymptomatic leaves on the same branches may also be submitted with symptomatic ones.

If you have access to a camera, take a photograph of the symptomatic region of the tree. Also, take photographs of the entire tree, being sure to include the symptomatic region in the photograph.

Fruit

Since fruit symptoms are far more indicative of CG than foliar symptoms, you should carefully examine any fruit that may be present on the affected branches. If any fruit showing CG symptoms are found, several typical symptomatic fruit should be submitted with the leaf and twig samples. Fruit is **not** known to harbor high concentrations of the bacterium, so it should **not** be sampled without accompanying leaf or stem tissue from the same tree.

Handling and Shipping

Pack leaves between dry paper towels and bundle the twigs together with a rubber band, then place samples in resealable plastic bags along with their identification. Double bag the sample and place the sample information on a sheet in the outside bag.

Fruit samples should be wrapped in dry paper towels and placed in paper bags, along with their identification. Double bag the sample. Keep the samples as cool as possible, and ship them via overnight delivery to the laboratory. Call the laboratory and advise them that samples are being shipped.

Records

Mark the tree with the sample identification, draw a map of its location on the property, and record GPS coordinates since tags are easily lost.

If Most Trees Show Symptoms

If most (or all) citrus trees on the property show symptoms throughout the tree, it may indicate a mineral deficiency problem. Submit samples from several of the trees exhibiting typical symptoms. Any trees with symptoms confined to one or a few branches, rather than the entire tree, should be sampled.

Citrus Psyllids

Trees should also be checked for the presence of psyllid vectors, particularly if new growth is present. If small pit galls are seen on young leaves, African citrus psyllids may be present. Samples of this psyllid should be collected and submitted for identification, regardless of which state the African psyllids are found in. Mark the tree the psyllids were collected from, and record its location using GPS.

Note the presence or absence of orange jasmine on the property, and if present check for Asian citrus psyllids. If Asian citrus psyllids are found in any states other than Florida or Texas, submit samples for identification of psyllids and the presence of *Ca*. Liberibacter spp. **See Identification of Psyllids on page D-1** and **DNA Extraction and PCR Detection in Citrus on page E-1** for more information.

Commercial Groves

Walk every row in the orchard in order to inspect as much of the newer growth and young branches on the outside of the trees as possible. Examine newer foliage and branches on larger trees, since older foliage is frequently spotted by other pathogens or covered with sooty mold, which can make it difficult to see CG symptoms.

Important Follow sanitation precautions to avoid spreading plant disease. **See Sanitation on page 3-2** and **Disinfecting Equipment on page F-1** for more information.

Important Reschedule inspections for commercial orchard blocks where harvesting operations are underway. Ask the owner or manager if any other cultural operations (e.g., hedging, irrigating, or fertilizing) are planned that might interfere with the inspection, and reschedule if necessary. Surveys should be scheduled for times when the citrus orchards are producing new foliage. Determine when the most likely season for flush of new leaves occurs in your area and try to target that time for surveys. Also check all sentinel trees in the areas adjacent to commercial groves.

Twigs, Leaves and Petioles

If symptoms are found on a tree, collect samples from those areas of suspect trees that show the best symptoms. Collect green twigs (6—8 inches long) with leaves and petioles attached. Some asymptomatic leaves on the same branches may also be submitted with symptomatic ones.

If you have access to a camera, take a photograph of the symptomatic region of the tree. Also, take photographs of the entire tree, being sure to include the symptomatic region in the photograph.

Fruit

Since fruit symptoms are far more indicative of CG than foliar symptoms, you should carefully examine any fruit that may be present on the affected branches. If any fruit showing CG symptoms are found, several typical symptomatic fruit should be submitted with the leaf and twig samples. Fruit is **not** know to harbor high concentrations of the bacterium, so should **not** be sampled without accompanying leaf or stem tissue from the same tree.

Handling and Shipping

Pack the leaves between dry paper towels and bundle the twigs together with a rubber band, then place samples in resealable plastic bags along with their identification. Double bag the sample and place the sample information on a sheet in the outside bag.

Wrap fruit in dry paper towels and place in paper bags, along with their identification. Double bag the sample and include collection information on a sheet inside the bag.

Keep the samples as cool as possible, and ship them via overnight delivery to the laboratory. Call the laboratory and advise them that samples are being shipped.

Records

Mark the tree with the sample identification number, and draw a map of its location within the block and the location of the block on the property. It may be helpful to designate one corner of the block—for example, NE corner—and count the number of rows from that corner and the number of trees down the designated row to precisely locate the sampled tree, or record the appropriate GPS coordinates. Flagging the sampled branch or branches will make it easier for subsequent sampling.

Citrus Psyllids

Trees should also be checked for the presence of psyllids, particularly if new growth is present. If small pit galls are seen on young leaves, African citrus psyllids may be present. Samples of this psyllid should be collected and submitted for identification, regardless of which state the African psyllids are found in. **See Identification of Psyllids on page D-1** for more information. Mark the tree the psyllids were collected from and map its location.

If Asian citrus psyllids are found in any states other than Florida or Texas, samples should be collected and submitted for identification. **See Survey Procedures on page 3-1** for more information. Note the position of any sample with GPS coordinates.

Nurseries

Citrus nurseries generally employ one or more of the following methods to grow trees:

- Outdoors in rows in the ground;
- Outdoors in containers (may be under shade cloth or in a lath house);
- Fully enclosed in a screenhouse, generally in containers; and
- Fully enclosed in a greenhouse, generally in containers.

Trees in rows in the ground are usually budded sequentially by budders moving down the rows. This is often **not** true of container-grown trees, which are moved about as nursery operations dictate.

Important Follow sanitation precautions to avoid spreading plant disease. **See Sanitation on page 3-2** and **Disinfecting Equipment on page F-1** for more information.

Important Nursery stock may be widely disseminated throughout a large geographic are—especially stock purchased by big retail chains—making it very important to thoroughly inspect all host trees being grown in the nursery.

Depending on tree and row spacing, it may be necessary to walk every row in order to see all of the foliage on each tree. Examine newer foliage and branches on larger trees, since older foliage is frequently spotted by other pathogens which may make it difficult to see CG symptoms. Any budwood source trees located at the nursery should also be inspected.

Twigs, Leaves and Petioles

Collect green twigs (6—8 inches long) with leaves and petioles attached. Some asymptomatic leaves on the same branches may also be submitted with symptomatic ones.

Fruit

Since fruit symptoms are far more indicative of CG than foliar symptoms, you should carefully examine any fruit that may be present on the affected branches. If any fruit showing CG symptoms are found, several typical symptomatic fruit should be submitted with the leaf and twig samples. Fruit is not know to harbor high concentrations of the bacterium, so should not be sampled without accompanying leaf or stem tissue from the same tree.

Handling and Shipping

Pack the leaves between dry paper towels and bundle the twigs together with a rubber band, then place samples in resealable plastic bags along with their identification and accurate GPS coordinates. Double bag the sample and place the sample information on a sheet in the outside bag.

Wrap fruit samples in dry paper towels and place in paper bags, along with their identification and accurate GPS coordinates. Double bag the sample. Keep the samples as cool as possible, and ship them via overnight delivery to the laboratory. Mark the samples and the package to indicate that they are nursery samples. A hold will be placed on the nursery until the samples are can give these samples priority handling. Call the laboratory and advise them that nursery samples are being shipped.

Records

Flag the sampled tree and attach the appropriate identifying numbers to it, as well as mapping its location within the block and the nursery and acquire accurate GPS coordinates.

High Suspect Samples

If high suspect samples are obtained, instruct the nursery owner or manager not to move nursery stock out of or within the property until results are obtained. (**See Rating of Suspect Plant Tissue Samples on page 4-6** for more information on sample rating.) Several suspect trees occurring sequentially in a row of young field-grown stock suggests the possibility that they were propagated from infected budwood. Question nursery personnel to determine the source of the budwood used to bud the stock and examine the tree if present. Based on this finding, conduct additional trace forward-traceback investigations if appropriate.

Citrus Psyllids

Trees should also be checked for the presence of psyllids. If small pit galls are seen on young leaves, African citrus psyllids may be present. Samples of the psyllids should be collected and submitted for identification. **See Identification of Psyllids on page D-1** for more information.

Note the presence or absence of orange jasmine on the property, and if present check for Asian citrus psyllids. If Asian citrus psyllids are found in any states other than Florida or Texas, samples should be collected and submitted for identification.

Mark the tree the psyllids were collected from and map its location and obtain accurate GPS coordinates.

4

Citrus Greening Disease

Chapter 4

Identification

Contents

Introduction

Accurate identification of citrus greening disease (CG) is pivotal to assessing its potential risk, developing a survey strategy, and determining the level and manner of control. Identification of the psyllid vectors is especially important in a new area where the disease is not known to occur. Increased surveillance for the disease may be undertaken in these areas once a vector is confirmed there.

Consult **DNA Extraction and PCR Detection in Citrus on page E-1** for details on approved diagnostic methods for the pathogens that cause CG.

Authorities

A USDA-recognized, centralized national survey screening center must positively identify the first suspected pest before initiation of survey and control program quarantine activities. Subsequent identifications may be performed elsewhere. For details, see **Centralized National Survey Screening Laboratory on page 4-2**.

PPQ permit and registration requirements for plant diseases and laboratories fall under the following two authorities:

- Plant Protection Act (7 CFR Part 330) (**See Plant Protection Act on page 4-2**)
- Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331) (**See Agricultural Bioterrorism Protection Act on page 4-2**)

Laboratories receiving suspect infected plant material or cultures are required to have PPQ permits. Laboratories possessing, using, or transferring select agents are required to be registered. However, diagnostic laboratories that identify select agents are exempt from this requirement as long as they complete APHIS–Centers for Disease Control and Prevention (CDC) Form 4

and destroy or transfer infected material to a laboratory registered with the APHIS Select Agent Program within the mandatory seven days. APHIS–CDC Form 4 is available at the Select Agent Web site.

Address

Agricultural Select Agent Program:
http://www.aphis.usda.gov/programs/ag_selectagent/index.html
Telephone: (301) 734-5960

Centralized National Survey Screening Laboratory

A National survey conducted in citrus growing states requires consistency in diagnostic procedures, and assurances that requirements for handling select agents are followed. During a survey, centralized molecular diagnostic screening tests must be conducted for all CG host material rated as high or medium suspect for CG. (For more information on rating, see **Rating of Suspect Plant Tissue Samples on page 4-6**.)

Until a CG proficiency test panel and laboratory approval program is implemented, all suspect CG plant and vector samples collected in the APHIS-funded National survey must be sent to the PPQ–National Identification Services–Molecular Diagnostics Laboratory (MDL).

Address

Dr. Mary E. Palm
USDA–APHIS–PPQ–National Identification Services
Molecular Diagnostics Laboratory
BARC-East, Bldg. 580
Powder Mill Rd.
Beltsville, MD 20705
T: 301-504-7154 or 504-5700 ext. 327
F: 301-504-6124
Email: mary.palm@aphis.usda.gov

See **Procedure for Sampling and Identification on page 4-4** for more information.

Plant Protection Act

Plant Protection Act permit requirements apply to all plant pests and infected plant material, including diagnostic samples, regardless of their quarantine status. If any material is shipped interstate, the receiving laboratory must have a permit. For further guidance on permitting of plant pest material, consult PPQ Permit Services or visit the PPQ Permits Web site.

Address

PPQ Permits Web site: http://www.aphis.usda.gov/plant_health/permits/
Telephone: (301) 734-8758

Agricultural Bioterrorism Protection Act

Federal regulation on Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331) specifies requirements for possession, use, and transfer of organisms listed as select agents and toxins. Once an unregistered diagnostic laboratory identifies a select agent, they must take the following steps immediately:

1. Notify the APHIS Agriculture Select Agent Program,
2. Complete and submit APHIS/CDC Form 4 within 24 hours, and
3. Destroy or transfer the agent to a registered entity within 7 days.

In compliance with this Act, if a diagnostic laboratory held back part of a screened sample for voucher purposes and that sample (forwarded to the PPQ–Molecular Diagnostics Laboratory) came back as positive for a select agent, the diagnostic laboratory is required to notify staff in the APHIS Select Agent Program immediately.

If the determination of the unregistered laboratory is to destroy the sample, this must take place within seven days of results notification and a PPQ Officer must witness the destruction of the sample on or before the seven-day period expires. Clarification of this and other information related to adherence to the select agent regulations is available on the APHIS Agricultural Select Agent website.

Address Agricultural Select Agent Program: http://www.aphis.usda.gov/programs/ag_selectagent/index.html
Telephone: (301) 734-5960

Procedure for Sampling and Identification

Samples of plants exhibiting CG symptoms must be submitted through normal regulatory networks. Follow the instructions below.

Instructions

1. Field personnel who are participating in a National survey will collect samples of plants that exhibit CG symptoms, and then submit the samples to a regulatory laboratory through their usual network. See **Symptoms On Citrus on page C-1** for images of symptoms to look for.

Sampling & Shipping Instructions

- Collect leaves with mid-ribs that are attached to stems.
- Leaf samples
  1. Send a minimum of 20 leaves with stems.
  2. Place leaf and stem samples with paper towels in two resealable plastic bags, one bag inside another larger bag.
  3. Express air from bag.
  4. Seal bag.
  5. Record sample identification number on bag.
  6. Keep samples cool, but **not** frozen (in an ice chest).
- Fruit samples
  1. Place fruit in two paper bags, one bag inside another larger bag.
  2. Record sample identification number on bag.
  3. Keep samples cool but **not** frozen (in an ice chest is good).
- Pack resealable bags inside a sturdy cardboard box. Include packing material to prevent movement in the box. Omit ice packs.
- Assign and record for each sample a unique ID sample number. Assure that the sample is linked to any survey data collected for that sample by including the Survey ID number on the form.
- Include the completed **PPQ 391 Specimens For Determination on page B-2**—and any relevant tags or barcodes that came with the sample—inside the outer bag.
- Do **not** send fruit samples without accompanying suspect leaf samples from the same tree.
- Use overnight delivery to ship samples; FedEx® is preferred. Send samples on the same day they are collected, or before noon the following day. Ship samples Monday through Thursday only.

2. Regulatory laboratory will use Table 4-1, "Decision table to determine rating of sample," on page 6 to perform a diagnostic test to rate samples as high, medium, or low suspect for CG.
3. Regulatory laboratory will forward all high and medium suspect leaf and stem samples to PPQ–National Identification Services–Molecular Diagnostics Laboratory (MDL).

Address

Dr. Mary E. Palm
USDA–APHIS–PPQ–National Identification Services
Molecular Diagnostics Laboratory
BARC-East, Bldg. 580
Powder Mill Rd.
Beltsville, MD 20705
T: 301-504-7154 or 504-5700 ext. 327
F: 301-504-6124
Email: mary.palm@aphis.usda.gov

Instructions

- Do **not** forward fruit samples without accompanying suspect leaf samples from the same tree.
- Mark packaging for all CG survey samples as follows: ATTENTION APHIS CITRUS SAMPLES

4. MDL will conduct molecular diagnostic screening tests on all samples that rate as high or medium suspect, with results within 24 hours after receipt. If a presumptive positive sample is determined, MDL will notify the originating state and PPQ.

Address

Dr. Mary E. Palm
USDA–APHIS–PPQ–National Identification Services
Molecular Diagnostics Laboratory
BARC-East, Bldg. 580
Powder Mill Rd.
Beltsville, MD 20705
T: 301-504-7154 or 504-5700 ext. 327
F: 301-504-6124
Email: mary.palm@aphis.usda.gov

Important

Do **not** send samples directly to MDL unless given specific instructions to do so.

Rating of Suspect Plant Tissue Samples

Regulatory laboratories will use Table 4-1, "Decision table to determine rating of sample," on page 6 to perform a diagnostic test to rate samples as high, medium, or low suspect for CG.

TABLE 4-1 Decision table to determine rating of sample

If symptoms include the following:	Then apply this suspect rating for CG:
Classic CG mottle[1] alone or accompanied by one or more of the following symptoms: ◆ Zinc-like deficiency ◆ Yellow veins ◆ Corking veins ◆ Misshapen or oddly colored fruit	High
Non-classic mottle[2] alone or in combination with the following symptoms: ◆ Yellow veins ◆ Vein corking ◆ Chlorotic leaves ◆ Zinc deficiency	Medium
◆ Zinc and other general deficiencies ◆ Mottling resulting from insect injury, fungal diseases, and mechanical damage to leaves ◆ Naturally senescing leaves ◆ Genetic variegation	Low

1 Classic CG mottle is usually visible on both leaf surfaces and mottling/discoloration passes through veins.

2 Non-classic mottle is visible only on adaxial surface and may or may **not** cross veins.

Psyllid Samples

If psyllids are forwarded for detection of the CG pathogens, make sure the insects are first correctly identified as citrus psyllids. This is especially important in states or counties where the citrus psyllid is **not** known to occur. After several citrus psyllids in an area have been confirmed, rely on local identifiers for verification of psyllid samples.

Psyllid samples taken for PCR analysis should be collected live and preserved in alcohol immediately. Psyllids that have been dead for more than two days (including those on sticky cards) will **not** yield accurate results when analyzed using PCR.

Identification of citrus psyllids carrying CG requires two steps:

Step 1. Confirm that the insect is a citrus psyllid.

Instructions

- Place the insects in a leak-proof vial containing 70% ethyl alcohol.
- Submit at least 3 insects from a location; more are preferable.
- Write the sample ID number in pencil on a label inserted into the vial.
- If the psyllid vectors of CG are **not** known to occur in the state, and suspect detection is made, fill out a separate PPQ Form 391 marked *Urgent* and forward to the Leader, Taxonomic Services Unit, Beltsville, MD. For instructions on completing PPQ Form 391, see **PPQ 391 Specimens For Determination on page B-2**.
- Some overnight carriers will **not** accept specimens manifested as preserved in alcohol, due to regulations on air transport of flammable materials. Ground transportation may be required.

Address

Leader, Taxonomic Services Unit
USDA–ARS–BA–PSI
Building 046, Room 101A, BARC-EAST
Beltsville, MD 20705-2350

Step 2. Conduct PCR analysis of citrus psyllids to detect CG.

Instructions

- Place the insects in a leak-proof vial containing 95% ethyl alcohol. The proper alcohol concentration is important for effective PCR analysis.
- Submit at least 3 insects; more are preferable.
- Write the sample ID number in pencil on a label inserted into the vial.
- If the psyllid vectors of CG are **not** known to occur in the state, and suspect detection is made from a site, for proper insect identification fill out a separate PPQ Form 391 marked *Urgent* and forward to the Leader, Taxonomic Services Unit, Beltsville, MD. For instructions on completing PPQ Form 391, see **PPQ 391 Specimens For Determination on page B-2**.
- Some overnight carriers will **not** accept specimens manifested as preserved in alcohol, due to regulations on air transport of flammable materials. Ground transportation may be required.

Address

Leader, Taxonomic Services Unit
USDA–ARS–BA–PSI
Building 046, Room 101A, BARC-EAST
Beltsville, MD 20705-2350

Sample Identification and Record Keeping

An electronic data collection system for survey and sample collection is currently being developed. Until the protocols for that system are finalized, complete a PPQ form 391. **See PPQ 391 Specimens For Determination on page B-2** for a copy of the form and instructions for completing it.

Inspectors must provide all relevant collection information with samples. This information should be communicated within a state and with the regional office program contact. If a sample tracking database is available at the time of the detection, please enter collection information in the system as soon as possible.

Web-Based Reporting Tool

A World Wide Web-based survey and diagnostic data collection and reporting tool for CG is in the development phase. When available, guidelines for data entry, access, and reporting will be provided by the PPQ Regional Program Manager.

State or Other Diagnostic Screening Laboratories Results

If a state, university, National Plant Diagnostic Network (NPDN), or private laboratory performs a PCR assay and detects a presumptive positive outside the national survey process outlined here, they must abide by the requirements under the Agriculture Bioterrorism Protection Act of 2002 (7 CFR 331). For more information, see **Agricultural Bioterrorism Protection Act on page 4-2**.

Diagnostic screening laboratories receiving samples are to communicate the date of receipt with their State Plant Regulatory Official and/or State Plant Health Director. All relevant sample information, and the diagnostic lab's determinations, must be communicated as soon as possible within a State and with the PPQ regional office program contact.

Approved Laboratory for Confirmatory Testing

Once the plant material has been screened and is known to be presumptive positive by molecular diagnostics, as soon as possible by overnight carrier forward the sample with the accompanying PPQ Form 391 to the USDA–APHIS–PPQ–Molecular Diagnostics Laboratory (MDL) in Beltsville, MD, for confirmation. MDL is authorized by APHIS to receive suspect domestic select agent plant pathogens under APHIS permit number 65253. A copy of the MDL permit for suspect domestic select agent pathogens need **not** accompany the package. **See PPQ 391 Specimens For Determination on page B-2** for more information on completing the form.

Potentially Actionable Suspect samples (PASS) in a program must be confirmed by MDL. MDL is the APHIS–PPQ–National Identification Services-recognized taxonomic authority for this pathogen. In the case of CG, the first presumptive positive(s) in a state are PASS samples. Presumptive positives in

counties outside of the initial positive county are also PASS samples. Any presumptive positive from a new host or other unexpected or unusual find must be treated as PASS samples.

The diagnostic laboratory with proper authorizations to do final confirmatory testing for *Candidatus* Liberibacter is the USDA–APHIS–PPQ–Molecular Diagnostics Laboratory (MDL) in Beltsville, MD. Notify MBL by email or fax that material is being sent; include the overnight service name and tracking number.

After the establishment of an emergency program, other laboratories may be certified and given authorizations to perform PPQ diagnostic tests.

Address

Dr. Mary E. Palm
USDA–APHIS–PPQ–National Identification Services
Molecular Diagnostics Laboratory
BARC-East, Bldg. 580
Powder Mill Rd.
Beltsville, MD 20705
T: 301-504-7154 or 504-5700 ext. 327
F: 301-504-6124
Email: Mary.Palm@aphis.usda.gov

Saturday Delivery to Molecular Diagnostics Laboratory

Do **not** send samples on Thursdays or Fridays because they may deteriorate over the weekend.

When approved by APHIS officials, samples may be sent on Thursdays or Fridays by Federal Express® because it is possible to have Saturday delivery by overnight carriers to the Beltsville facility. However, this must be determined by consultation and arrangement with APHIS. If you verify with APHIS officials that samples will be accepted on Saturday, the Federal Express® tracking number to the MDL in Beltsville must be provided by Friday no later than 2 PM EST by email so they can notify their local office to authorize Saturday delivery**.**

Notification of State Officials of Sample Submissions and Results

Notify the State Plant Health Director and State Plant Regulatory Officials in the sample state of origin and fax the PPQ regional office of any sample forwarding information, completed documentation, including overnight freight tracking information. Once results are known, States will be notified by the PPQ regional office of the results.

Do not contact the MDL to get sample results. This information will be reported through the appropriate and approved reporting lines to the regions and States from PPQ headquarters as soon as they are available. The MDL will direct any inquiries to PPQ headquarters.

Sample Processing Time

Growers and cooperators need to be aware that sample processing and testing time of at least 48 hours is required for the MDL. This is in addition to the time it takes to process and forward samples from the intermediate state or cooperating university diagnostic laboratories.

5

Citrus Greening Disease

Chapter 5

Regulatory Procedures

Contents

Instructions to Officers

Agricultural officers must follow instructions for regulatory control measures, treatments, or other procedures when authorizing the movement of regulated articles. A full understanding of the instructions and procedures is essential when explaining procedures to persons interested in moving articles affected by the quarantine and regulations.

Only authorized treatments may be used in accordance with labeling restrictions. **See Laws Pertaining to Pesticide Use on page 6-1** for more information.

During all field visits, follow proper sanitation procedures. For more information, see **Sanitation on page 3-2** and **Disinfecting Equipment on page F-1**.

Quarantine Actions and Authorities

After an initial suspect positive detection, an Emergency Action Notification PPQ Form 523 (EAN) can be issued to hold articles or facilities, pending positive identification by a USDA–APHIS–PPQ recognized authority or further instruction from the PPQ Deputy Administrator.

If necessary, the Deputy Administrator will issue a letter directing PPQ field offices to initiate specific emergency action under the Plant Protection Act of 2000 (Statute 7 USC 7701-7758) until emergency regulations can be published in the *Federal Register*.

Emergency Quarantine Action

The Plant Protection Act of 2000 provides for authority for emergency quarantine action. This provision is for interstate regulatory action only; intrastate regulatory action is provided under state authority.

State departments of agriculture normally work in conjunction with Federal actions by issuing their own parallel hold orders and quarantines for intrastate movement. However, if the U.S. Secretary of Agriculture determines that an extraordinary emergency exists and that the measures taken by the state are inadequate, USDA can take intrastate regulatory action provided that the governor of the state has been consulted and a notice has been published in the *Federal Register*. If intrastate action cannot or will not be taken by a state, the PPQ may find it necessary to quarantine an entire state.

Access to Private Property

PPQ works in conjunction with state departments of agriculture to conduct surveys, enforce regulations, and take control actions. PPQ employees must have permission of the property owner before entering private property. Under certain situations during a declared extraordinary emergency or if a warrant is obtained, PPQ can enter private property in the absence of owner permission.

PPQ prefers to work with the state to facilitate access when permission is denied; however, each state government has varying authorities regarding entering private property. A General Memorandum of Understanding (MOU) exists between PPQ and each state that specifies various areas where PPQ and the state department of agriculture cooperate. For clarification, check with your State Plant Health Director (SPHD) or State Plant Regulatory Official (SPRO) in the affected state.

Overview of Regulatory Program for Citrus Greening Disease

Once an initial U. S. detection is confirmed, holds will be placed on the property by the issuance of an EAN. Immediately place a hold on the property to prevent the removal of CG host plants or budwood, or hosts of the psyllid vectors. This should include both citrus and non-citrus host plants, such as orange jasmine *(Murraya paniculata)*.

There is no need to place a hold on fruit, since it poses no risk of pathogen movement to other trees, provided care is taken to prevent inadvertently moving psyllid vectors with the fruit.

Results of traceback and trace forward investigations (see **Survey Procedures on page 3-1**) from the property will determine the need for subsequent holds for testing or taking further regulatory actions. Further delimiting surveys and testing will identify positive properties requiring holds and regulatory measures prescribed.

Record Keeping

Record keeping and documentation are important for holds and subsequent actions:

- Rely on receipts, shipping records and information provided by homeowners, the grower, farm manager, or nursery manager;
- Document the following:
  - Origin and destination for shipped plant material,
  - Movement of plant material within the facility, and
  - Cultural or sanitation practices employed.
- Record the quantity and type of plant material held, destroyed, or requiring treatment in control actions;
- Consult a master list of properties distributed with the lists of suspect nurseries based on traceback and trace forward investigations, or nurseries within a quarantine area;
- Draw maps of the facility layout to suspect plants and other potentially infected areas;
- When appropriate, take photographs of the tree symptoms property layout;
- Document plant propagation methods, labeling, and any other situation that may be useful for further investigation and analysis; and
- Keep all written records filed with the EAN copies, including copies of sample submission forms, documentation of control activities, and related state issued documents if available.

Issuing an Emergency Action Notification

Find EAN PPQ Form 523 (EAN) in **Appendix B on page B-1**. The form is also available electronically at the APHIS Web site.

Address	http://www.aphis.usda.gov/library/forms/pdf/ppq523.pdf

Issue an EAN in order to hold all host plant material at facilities that have the suspect plant material directly or indirectly connected to positive confirmations. Once an investigation determines the plant material is **not** suspect, or testing determines there is **no** risk, the material can be released and the release documented on the EAN.

The EAN can also be issued to hold plant material in fields pending positive identification of suspect samples. Use the same EAN to document actions taken (such as destruction and disinfection) in the following circumstances:

- Decision to destroy plants was made, and
- Receipt of submitted samples was confirmed.

Additional quarantine action may be warranted in the case of groves that test positive for CG pathogens.

If plant lots or shipments are to be held as separate units, issue separate EANs for each held unit of suspect plant material associated with that unit. EANs are issued under the authority of the Plant Protection Act of 2000 (statute 7 USC 7701-7758). It is advised that states issue their own hold orders parallel to the EAN to ensure that plant material cannot move intrastate.

When using EANs to hold articles, clearly specify the actions to be taken. An EAN issued for positive testing and positive associated plant material must clearly state that the material must be disposed of, or destroyed, and areas disinfected. State that these actions will take place at the owner's expense, and will be supervised by a regulatory official. If the EAN is used to issue a hold order for further investigations and testing of potentially infested material, then be sure to document—on the same EAN—all disposal, destruction, and disinfection orders resulting from investigations or testing.

Regulated Articles

Once initial detections are confirmed in an area[1], regulated articles include all live host plant material in that area. CG pathogens are spread by vectors and fresh propagative plant material harboring the bacteria within the phloem of the plant tissue. Whole live plants, stems, leaves, and cuttings are regulated.

Fruit is currently **not** considered a pathway for CG disease spread and so is **not** regulated. Because of recent scientific developments, seed of citrus and citrus relatives is now being regulated. However, precautionary fruit cleaning and/or inspections should be performed in areas where the psyllid vector occurs since the fruit may be a pathway for hitchhiking citrus psyllids.

Hosts of Asian Citrus Psyllid and *Candidatus* Liberibacter

Hosts of *Candidatus* Liberibacter spp. and the Asian citrus psyllid, included in **Table 5-1**, are prohibited movement outside the quarantine area. If one of the psyllid vectors has been confirmed in the state, movement of the psyllid host plants included in **Table 5-2** are also restricted and subject to regulatory treatments.

TABLE 5-1 Regulated hosts of Asian citrus psyllid and *Candidatus* Liberibacter spp.

Scientific Name	Common Name
Aeglopsis chevalieri	Chevalier's aeglopsis
Balsamocitrus dawei	Uganda powder-flask-fruit
Calodendrum capense	Cape-chestnut
X Citrofortunella microcarpa	calamondin
X Citroncirus webberi	citrange
Citrus spp.	sweet orange[1], mandarine orange[1], sour orange[2], lemon[2], grapefruit[2], tangerine, pomelo[2]
Clausena indica	clausena
Clausena lansium	wampee, wampi
Fortunella spp.	kumquat
Limonia acidissima	Indian wood-apple
Microcitrus australasica	finger-lime
Murraya koenigii	curryleaf
Murray paniculata	orange jasmine
Poncirus trifoliata	trifoliate-orange
Severinia buxifolia	Chinese box-orange
Swinglea glutinosa	tabog
Toddalia lanceolata	toddalia
Triphasia trifolia	trifoliate limeberry

1 Highly susceptible

1 Positive county or predetermined buffer area around positive finds after a thorough delimiting survey

2 Moderately susceptible

Hosts of Asian Citrus Psyllid and Not Citrus Greening

If one of the psyllid vectors has been confirmed in the state, movement of the psyllid host plants included in **Table 5-2** are also restricted and subject to regulatory treatments. Plants included in **Table 5-2** are hosts of the Asian citrus psyllid, but have **not** been shown to be hosts for *Candidatus* Liberibacter spp. They are important as reservoirs or potential pathways for infected insect vectors to non-infested citrus growing states and should be regulated.

TABLE 5-2 Hosts of Asian citrus psyllid and not *Candidatus* Liberibacter spp.

Scientific Name	Common Name
Aegle marmelos	bael, Bengal-quince
Afraegle gabonensis	Gabon powder-flask-fruit
Afraegle paniculata	Nigerian powder-flask-fruit
Atalantia spp.	atalantia
Citropsis gilletiana	Gillet's cherry-orange
Citropsis schweinfurthii	West African cherry-orange
Clausena anisum-olens	anis
Clausena excavata	clausena
Eremocitrus glauca	Australian desert-lime
Eremocitrus hybrid	desert-lime
Merrillia caloxylon	katinga
Microcitrus australis	Australian round-lime
Microcitrus papuana	Brown River finger-lime
X Microcitronella 'Sydney'	faustrimedin
Naringi crenulata	hesperethusa
Pamburus missionis	pamburus
Toddalia asiatica	orange-climber
Vepris lanceolata	white ironwood
Zanthoxylum fagara	wild-lime

Shipment of Hosts of Asian or African Citrus Psyllid

Nurseries in states with confirmed detections, of either Asian or African citrus psyllid, that wish to ship any insect vector hosts (**not** hosts of CG) from a regulated property or quarantined area to non-citrus growing states, must do so under a limited permit and a compliance agreement. The agreement requires prescribed regulatory treatments (**See Regulatory Treatments For Nurserystock Hosts of Psyllid Vectors on page 5-7**) to eliminate the risk of spreading live insect vectors. Citrus growing states **without** established CG infections normally will be prohibited destinations for psyllid hosts regardless of treatments applied.

Regulatory Treatments For Nurserystock Hosts of Psyllid Vectors

In citrus growing states where African or Asian citrus psyllids are known to occur within CG positive quarantined counties, or within established buffer areas around positive tree finds, the psyllid host plants included in **Table 5-2** are eligible to move out of a quarantine area after regulatory treatments are applied.

Drench plants with an appropriately labeled systemic insecticide, followed by a foliar spray at specified time periods prior to shipment. All articles regulated as psyllid hosts must be treated with a drench containing imidacloprid as the active ingredient. In addition, 10 days prior to movement, plants must receive a foliar spray with a product containing either acetamiprid, chlorpyrifos, or fenpropathrin, as the active ingredient. The treatments will be followed by a visual inspection for living psyllids within 72 hours prior to certification and shipping.

Example For example, drench all plants regulated as psyllid hosts with Marathon 60 WP, thirty days prior to movement. In addition, 10 days prior to movement, apply a foliar spray of Tame EC, Dursban E, Discus, or Tristar, at label rates.

For more information on labels and available products, see **Insecticides on page G-1**.

Quarantine Area

In states **without** confirmed presence of one of the citrus psyllid vectors regulatory officials, in consultation with an CG science panel, should consider a buffer distance around known positive detections in which to regulate nursery stock.

In states where the presence of psyllid vectors has been confirmed, once a positive disease detection is confirmed, all nurseries with disease and psyllid host material within an agreed upon buffer area are subject to quarantine and

adherence with the prohibition of shipping CG hosts (**Table 5-1**) outside the quarantined area and the psyllid hosts (**Table 5-2**) without required regulatory treatments.

TABLE 5-3 Decision table to determine buffer area

If in a state where the presence of psyllid vectors is:	And a positive detection of CG is:	Then:
Not confirmed	→	In consultation with an CG science panel, consider a buffer distance around known positive detections in which to regulate nursery stock.
Confirmed	Confirmed	All nurseries with disease and psyllid host material within an agreed upon buffer area are subject to quarantine and adherence with the prohibition of shipping CG hosts (**Table 5-1**) outside the quarantined area and the psyllid hosts (**Table 5-2**) without required regulatory treatments.

Grower Requirements Under Quarantine

Depending on decisions made by Federal and state regulatory officials in consultation with an CG science panel, quarantine areas may have certain other requirements for groves, nurseries, or residential properties in that area, such as tree removal and destruction, psyllid control measures, or plant waste material disposal.

Any insecticides used to control psyllids, or herbicides used to treat plants, will be labeled for that use or exemptions will be in place to allow the use of other materials.

Nurseries within quarantine areas and other at risk areas are required to screen all outdoor host plant propagation areas to keep areas free of psyllid vectors.

Establishing a Federal Quarantine

Regulatory actions undertaken using EANs continue to be in effect until the prescribed action is carried out and documented by regulatory officials. Regulatory actions can be short-term destruction or disinfection orders or longer term requirements for growers that include prohibiting the planting of host crops for a period of time. Over the long term, producers, shippers, and processors may be placed under compliance agreements. Permits may be issued to move regulated articles out of a quarantine area or property under an EAN.

Results analyzed from investigations, testing, and risk assessment will determine the area to be designated for a Federal and parallel state quarantine. Risk factors will take into account positive testing, positive associated, and potentially infested exposed trees. Boundaries drawn can include a buffer area determined based on risk factors and epidemiology.

PPQ may issue a Federal Order, followed by an interim rule establishing a quarantine to be published in the *Federal Register*, which is normally drafted by PPQ headquarters staff in consultation with the region, SPHD, and SPRO. The conditions that growers must abide by within a quarantine area are included in the rule. Regulated articles and conditions allowing movement of articles out of the regulated area are determined and included in the regulation, along with other administrative requirements.

Removing Areas From Quarantine

If investigations determine the quarantine restrictions on fields are adhered to over the prescribed time periods, and actions are documented, fields can be released from quarantine restrictions. Notify growers that their fields may be subject to additional monitoring by state or Federal officials for the presence of CG pathogens.

Regulatory Records

Maintain standardized regulatory records and database(s) in sufficient detail to carry out an effective, efficient, and responsible regulatory program.

Use of Chemicals

The PPQ *Treatment Manual* and this *Guidelines* identify the authorized chemicals, and describe the methods, rates of application, and special instructions. See **Control on page 6-1** for more information. Concurrence by PPQ is necessary before using any other chemical or procedure for regulatory purposes.

6

Citrus Greening Disease

Chapter 6

Control

Contents

Introduction

USDA–APHIS–Plant Protection and Quarantine (PPQ) develops and makes control measures for citrus greening disease (CG) and its vectors available to involved states. Environmental Protection Agency (EPA) approved and labeled treatments will be recommended when available.

Laws Pertaining to Pesticide Use

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) authorizes the Environmental Protection Agency (EPA) to regulate pesticides. All persons using and applying pesticides should understand the laws pertaining to pesticide use and application. The following are provisions of FIFRA that are most pertinent to emergency pest control programs:

- Restricted use pesticides must be applied by a certified applicator;
- Use of any pesticide inconsistent with the label is prohibited; and
- Violations can result in heavy fines or imprisonment.

States can register pesticides on a limited basis for special local needs according to the following Sections:

- **Section 18**—EPA administrators can exempt Federal or state agencies from FIFRA if it is determined that emergency conditions exist that require such exemptions; and
- **Section 24**—A state can provide registration for additional uses of Federally registered pesticides formulated for distribution and use within that state to meet special local needs in accordance with the purposes of this act.

For additional information concerning exemptions, see the Emergency Programs Manual, Section 14. Contact Environmental Services staff to assure that any pesticide being considered as part of an eradication program conforms to pesticide use requirements. Obtain all required environmental documentation before beginning.

Control Decisions and Oversight

Important All quarantine actions related to destruction are to be witnessed, supervised, and documented by a Federal and/or state plant regulatory official whenever possible. Because some strains of CG are listed as select agents under the Agriculture Bioterrorism Protection Act of 2000, proper supervision and documentation of destructions of infected plant material is critical. If a PPQ representative is **not** available, a state cooperating inspector can witness and document the disposal.

If a property is infected with a CG pathogen, known infected trees are to be removed to eliminate them as inoculum reservoirs. After assessment and an effective delimiting survey, control or containment of the disease may include removal and destruction of exposed plants within buffers. Buffer distance is difficult to determine for the following reasons:

- Infections can be latent in a plant; symptoms can appear six months to two years after infection,
- Diagnostic tools are inadequate, and
- Dispersal distance for the psyllid vectors is unknown.

Recommendations from the CG Science Panel may include removal of entire citrus blocks in groves with known infections and psyllids.

If there is a confirmed detection in an area with psyllid populations, control strategies will be determined by Federal and state regulatory officials in consultation with the science advisory panel consisting of members knowledgeable of the disease and its epidemiology. Likewise, insect vector control decisions will also be made based on the type of property infested in consultation with the science advisory panel.

Control Records

Maintain a record of control actions; include maps. Retain all documentation, receipts, or other pertinent records. Attach documentation to the office copy of the **PPQ 523 Emergency Action Notification on page B-5**. Include the following information:

- Locations of all detections,
- Number and type plants subjected to control actions, and
- Materials and formulations used in each treated area.

Environmental Monitoring

Environmental monitoring is an important consideration in all programs. Contact PPQ headquarters to learn if environmental monitoring or assessment are required. Environmental Services staff can evaluate environmental impact by monitoring the following:

- Air, to detect residual airborne pesticides;
- Water, to detect insecticide levels resulting from direct application, leaching, and runoff;
- Soil, to determine insecticide levels and residues;
- Foliage, to detect residues; and
- Non-target organisms before, during, and after applications and post treatments, to determine impact of pesticide.

Citrus Health Response Program

See the Citrus Health Response Program (CHRP) Web site to learn more about the minimum standards for citrus health in the United States. The Program includes recommendations and a regulatory component that includes long-term management practices for CG while maintaining production and commerce. The procedures developed as a part of that process will provide further guidelines on CG disease management.

Address	http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/index.shtml

Control of Citrus Greening Disease

Remove and destroy infected trees, since CG cannot be eliminated. Treatment with antibiotics suppresses CG symptoms; however, it is impractical, phytotoxic, and will **not** eliminate the bacteria from the tree.

Various methods have been attempted in other countries to control infestations of CG in citrus; however, the disease has **not** been successfully eradicated or suppressed in the short-term. While long-term management techniques are available and are being investigated, new detections require quick action. The following guidelines can minimize the spread and reduce the incidence of infection in an area if caught early.

No Known Citrus Psyllid Vectors Present

Because CG is a self-limiting bacterial disease spread only by the vector or grafting, it is likely that a positive detection in an area where the psyllid vector is **not** known to occur will represent an isolated infection. The infected plant may have been illegally imported from a country or state with CG and planted there, or grafted from such a plant.

Take the following steps to control CG where **no** known citrus psyllid vectors are present:

- Trace all budwood or other grafting sources associated with positive detections from commercial groves or nurseries, and conduct thorough traceback and trace forward investigations for positive trees from residential areas;
- Collect and test any plant of the same species (as the infected plant) in the area in case it was grafted from the same source of infected material;
- Remove and destroy all known infected plants and suspect associated host plants that may have come from the same budwood source, even if they are symptomless; and
- Place under quarantine nurseries or other establishments with sources of budwood or trees that were traced from positive detections. Do **not** allow shipping of host material from those properties or from a quarantined area. Suspect properties with associated grafting from infected sources can be quarantined until a thorough investigation determines the level of risk for those properties shipping propagative material out of the area.

Citrus Growing States or Areas with Known Citrus Psyllid Infestations

In citrus growing areas where the vector is present, effective early detection and eradication are **unlikely** for the following reasons:

- Infections can be latent in a plant; symptoms can appear six months to two years after infection; and
- Diagnostic tools for detecting latent infections are **not** sensitive enough to allow confident delineation of the infection in an area.

If the overall severity of the disease in a geographic area can be assessed and determined to be low or isolated, removal of infected trees will eliminate sources of inoculum. Removal of exposed trees within a buffer area is **not** practical at this time.

If effective tools were available and validated, an aggressive eradication program would involve removing exposed trees around trees that test positive in a large buffer area. The size of the buffer area depends on epidemiology of the disease and vector's dispersal ability. Dispersal of the African citrus psyllid is thought to be approximately 1.5 kilometers. However, only anecdotal information on the Asian citrus psyllid is known. Dispersal behavior may be density dependent. Psyllids may be carried long distances on wind currents.

Control of Psyllid Vectors

Use chemical and biological control methods to control the African and Asian citrus psyllids. A site-specific Environmental Assessment may be required prior to the initiation of a control program. **See Environmental Assessments on page 7-3** for related information.

Chemical Control

Several insecticides have been shown to provide effective control of the citrus psyllids in nursery stock. **See Insecticides on page G-1** for more information on insecticides. Prior to implementation, review method and rate of application for larger trees in mature groves with the Huanglongbing Science Panel. **See Citrus Greening Science Panel on page -ii** for a list of members.

Use visual inspection, or install yellow sticky cards, to monitor psyllid populations and determine the best time to apply an insecticide. Be aware of population trends of psyllids in infested areas before application. Populations build up during flushing periods and may also be influenced by populations on nearby ornamental hosts. Densities may also be higher at the edges of groves.

Control citrus psyllids prior to destroying infected trees, to minimize their spread. This prevents dispersal of infected adults during tree cutting operations.

Before Application

Before applying insecticides, assure the proper environmental documentation is in place and environmental monitoring has been arranged if appropriate. Coordinated outreach and notifications of quarantine actions will minimize opposition by landowners and residents. These efforts are to be closely coordinated with state agencies responsible for pesticide applications and licensing.

Biological Control

The Citrus Greening Science Panel does **not** recommend biological control of psyllid populations as an effective method of reducing the incidence of disease in an area. (For a list of members, see **Citrus Greening Science Panel on page -ii**.) However, in an area wide pest management program, biological control may be effective in reducing vector populations in residential areas, to the benefit of homeowners and commercial producers with groves adjacent to residential areas.

Biological control has been effective in several countries, using a variety of parasites and predators. Biological control of the two citrus psyllid vectors was achieved on Reunion Island (France: Indian Ocean) with hymenopteran psyllid parasites.

African Citrus Psyllid

Tamarixia dryi was introduced from South Africa against the African citrus psyllid.

Asian Citrus Psyllid

Tamarixia radiata was introduced from India against the Asian citrus psyllid. Parasitization of the psyllid reached 95% in North India, and 90% in South India. When the encyrtid *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agaral) was added, parasitization reached 92.5% on Reunion Island. Parasitization reached nearly 100% in Taiwan. The two parasites specific to the Asian citrus psyllid were introduced into Florida in 1999, but only *T. radiata* is established.

Other Biological Control Agents

Also in Florida, populations of the native ladybeetle *Olla v-nigrum* have increased significantly since the introduction of the Asian citrus psyllid, and this ladybeetle, together with the Asian multicolored ladybeetle (*Harmonia axyridis),* have been found preying on the Asian psyllid throughout its range in Florida. Fungi also attack the Asian psyllid in Florida's warm, moist climate. Biological control, however, may not be sufficient to adequately reduce insect populations, especially during the early spring months or in nurseries where trees are constantly putting on new growth.

Planning a Biological Control Program

Biological control of African and Asian citrus psyllids requires further investigation to be effective. The following guidelines should be followed when planning a biological control program:

1. Identify an effective biological control agent with the potential to control the target pest or pests.
2. Locate a reliable source of the biological control agents.
3. Determine the following:
   - ❖ Release rates
   - ❖ Synchronization with the host
   - ❖ Temperature and other environmental requirements
   - ❖ Appropriate host plants
4. Coordinate their introduction with other strategies in a pest management program. For example, identify compatible pesticides and cultural practices.

Control Procedures for Positive Nursery Detections

Use the following guidelines in nurseries if the first detection in an area occurs there, and evidence of a general infection in surrounding areas is absent. Take the following steps immediately upon confirmation that a citrus nursery sample is positive for CG:

Step 1 If **not** done at the time of sampling, an immediate quarantine hold must be placed on the nursery to prevent the movement of trees and budwood from the premises.

Step 2 Check nursery records to obtain names and addresses for all sales during the prior six months. These should be grouped in three sales categories:

- In the past month;
- In the two months prior to that; and
- In the three months prior to that, for a total of six months.

Step 3 Evaluate the disease situation, including identification and inspection of the budwood source(s) of the diseased tree(s). Consider the following:

- What is the source of the infected trees?
- Have plants from the same sources been distributed or sold to other areas?
- Within the nursery, how many trees show symptoms? What is the distribution of trees showing symptoms?
- Are the diseased trees (assuming more than one) clustered together or randomly scattered? If clustered, does it suggest possible use of infected budwood or (more likely) tree-to-tree movement of the vectors?
- Were any symptoms present on the budwood source tree(s)?
- Are vectors present and, if so, in what numbers?
- Has the nursery maintained a good vector control program?
- Are alternative (non-citrus) hosts of the vector (e.g., orange jasmine in the case of Asian citrus psyllid) present on or near the property?
- Are there any citrus trees on the properties immediately adjacent to the nursery and, if so, do any of the trees show symptoms of CG?
- Do the trees in the nursery appear to be healthy and growing vigorously?
- What grafting practices have taken place? What are the sanitary measures taken by propagators in the nursery?

Step 4 Commence vector control and tree removal operations as soon as possible.

Removal of Trees

In consultation with the Huanglongbing Science Panel, consideration should be given by state and Federal regulatory officials to removing all trees, depending on the size and physical layout of the nursery.

Remove all trees within the blocks plus any trees in adjacent blocks of with infected trees under the following conditions:

- Nursery is large, with trees set out in blocks and with some separation between blocks; and
- Infected trees are all within one or two blocks.

If vectors are known to occur in the area, all trees in the nursery should be treated with an approved insecticide prior to commencement of removal operations.

Follow-up Inspection

Following completion of the tree removal operations, the trees in the nursery should be inspected on a bi-weekly schedule for the next two months. If **no** additional positive trees are found, inspect on a monthly schedule for the remainder of the quarantine period. If the trees appeared healthy and vigorous, a six-month hold after the last positive is removed should be adequate for infected trees to show symptoms. Any infected trees found during subsequent inspections will reset the clock on the hold period.

Vector Control—The nursery should maintain a good vector control program during this period, including removal of alternative hosts of the vector present on the nursery property.

If the psyllid vector occurs in the area, citrus nurseries may be required to locate operations to greenhouses to protect from infective vectors.

After the Hold

If certain groups of trees would be beyond prime condition for sale at the end of the hold period, especially if it goes beyond six months, the nursery may voluntarily elect to destroy those trees to avoid further expenses involved with their maintenance. Small nursery operations may voluntarily choose to destroy all trees for economic reasons, rather than maintain them for the six-month (or longer) hold period.

Traceback Investigation

Traceback investigation of prior sales of nursery stock should begin as soon as possible, with initial survey directed at trees sold in the previous month. When completed, go to the second group, sales in the two months before that. If **no** CG-infected trees are found in either group, it may **not** be necessary to inspect the third group. If, however, positive trees are found, the third group should also be inspected.

Tree Removal and Disposal

Standard tree removal methods can be employed, making certain that the trees have been sprayed with an insecticide prior to removal, to kill any psyllid life stages which may be present. Physical removal of the trees can occur by pulling or pushing the tree out of the ground with heavy equipment. If this technique is used, plants may later sprout from roots left in the ground, and these must be controlled with an approved herbicide such as glyphosate. Trees can also be removed by cutting them at or near the soil line. If the latter method is employed, the freshly-cut stump should be treated with an herbicide such as Tordon® or Garlon® to kill it and minimize root sprouting. Check the label to be sure this use meets requirements.

Unlike many other citrus pathogens, CG bacteria are spread only by grafting with infected budwood and by the two psyllid vectors. Therefore, any method of disposal which kills any vectors present, prevents further access to foliage by vectors, and prevents usage of removed trees as budwood sources is appropriate. Suitable disposal methods include burning, chipping (only smaller diameter branches and foliage would need to be chipped; large diameter wood could be disposed of by other means), or burial in a landfill.

Control of Hosts of Insect Vectors

Another major component of an effective control program is the removal of preferred alternative hosts of the vectors. In the case of Asian citrus psyllid in Florida and Texas, this would involve the removal, for example, of any orange jasmine plants growing near citrus plantings, and especially any growing near citrus nurseries.

Nurseries that wish to ship any insect vector hosts from a regulated property to citrus growing states must do so under a compliance agreement that requires prescribed treatments to eliminate the risk of spreading the insect vectors. **See Regulatory Procedures on page 5-1** for more information.

Long Term Citrus Greening Management

If eradication or containment is **not** feasible, we may have to employ a management program similar to the program used in South Africa. This integrated approach may allow production to continue, and includes the following tactics:

- Remove alternative hosts of the vector throughout the production area, to minimize vector population carryover when citrus is **not** flushing;
- Create new plantings from a certified, disease-free program;
- Monitor groves to detect increasing populations of vectors, followed by prompt vector control measures; and
- Inspect groves to detect CG symptoms as early as possible, followed by prompt removal of trees.

7

Citrus Greening Disease

Chapter 7

Environmental Regulation

Contents

Introduction

A key element in designing a program or an emergency response is consultation with Environmental Services (ES), a unit of APHIS' Policy and Program Development Staff. ES prepares environmental documentation, such as environmental impact statements (EIS) and environmental assessments (EA), to aid in program operational decisions. ES also coordinates pesticide registration and approvals for APHIS pest control and eradication programs, ensuring that registrations and approvals meet program use needs and conform to pesticide use requirements. Refer to **Resources on page A-1** for additional information.

Disclaimer

All uses of pesticides must be registered or approved by appropriate Federal, state, and/or tribal agencies before they can be applied. The information provided on pesticide labels may **not** reflect all of the actual information, including precautions and instructions for use, which you are required to follow in your specific state or locality. It is the responsibility of persons intending to use a pesticide to read and abide by the label, including labeling that has been approved for the particular state or locality in which the chemical is to be used, and to comply with all Federal, state, tribal, and local laws and regulations relating to the use of the pesticide. APHIS program staffs are responsible for their compliance with applicable environmental regulations.

National Environmental Policy Act

Agencies should prepare an EA or EIS concurrently and integrated with environmental impact analyses, surveys, and studies required by the Fish and Wildlife Coordination Act, National Historic Preservation Act of 1966, Endangered Species Act, and other laws and executive orders. Environmental document prepared to comply with other acts also may be incorporated into National Environmental Policy Act (NEPA) documents as part of the NEPA process.

Categorical Exclusions

Categorical exclusions are categories of actions that do **not** have a significant effect on the quality of the human environment and for which neither an EA nor an EIS is generally required.

APHIS managers are encouraged to use categorical exclusions where appropriate to reduce paperwork and speed the decision making process. Proposed actions are subject to sufficient environmental review to determine whether they fall within the broadly defined categories. Each time a specific categorical exclusion is used, the required review must be done. An EA may be prepared for proposed actions otherwise excluded when the manager determines that the action may have potential to significantly affect the environment, or an EA would be helpful in planning or decision making.

Environmental Impact Statements

An EIS is a detailed statement that must be included in every recommendation or report on proposals for legislation and other major Federal actions significantly affecting the quality of the human environment. The primary purpose of an EIS is to serve as an action-forcing device to insure that the policies and goals defined in the National Environmental Policy Act (NEPA) are infused into the ongoing programs and actions of the Federal government. Generally, EISs are prepared when Federal agencies recognize that their actions have the potential for significant environmental effects (adverse or beneficial), or when an EA leads to a finding of potential significant impact.

APHIS prepares EISs for administrative proceedings that establish broad scale significant impact-generating strategies, methods, or techniques such as large-scale aerial pesticide applications. This can include contingency or emergency strategies that are comprehensive in scope or long-range plans with potential for significant environmental impact. APHIS also prepares programmatic EISs to examine strategies and options for dealing with issues with important implications for the maintenance and enhancement of environmental quality.

Environmental Assessments

An EA is a concise public document that briefly provides sufficient evidence and analysis for determining whether to prepare an EIS or finding of no significant impact (FONSI). An EA aids an agency's compliance with the National Environmental Policy Act (NEPA) when no EIS is necessary and facilitates the preparation of an EIS when necessary. Generally, an EA leads to a FONSI or an EIS, but it could also lead to abandonment of a proposed action.

The content of an EA must include brief discussions of the need, alternatives, and potential environmental impacts of the proposal a list of agencies and persons consulted.

Environmental Monitoring

PPQ requests assistance from ES before PPQ personnel or funding are used for control operations. Additionally, program staff should consult with the PPQ–Environmental Monitoring staff to determine if an environmental monitoring plan is required for the operation. State, regional, and national program managers determine counties where treatments may be needed.

Program personnel should evaluate the success of biological control agents and herbicide treatments used in eradication or suppression of the target FNW or host weeds and avoid damage to non-target plants.

Biological Assessment

A biological assessment (BA) is an analysis of the effects that a Federal agency action may have on listed or proposed endangered or threatened species and designated critical habitat. The Endangered Species Act (ESA) requires this analysis if the proposed action may affect a listed species. In such a case consultation with the U.S. Fish and Wildlife Service (FWS) or the National Marine Fisheries Service (NMFS) is required. Federal agencies are required to insure that any action authorized, funded, or carried out is not likely to jeopardize listed species or result in adverse modification of designated critical habitat.

Glossary

APHIS. Animal and Plant Health Inspection Service.

ARS. Agricultural Research Service.

blotchy mottling. Characteristic symptom of CG disease on citrus leaves caused by infection by *Candidatus* Liberibacter species. Symptoms appear on both sides of the leaf as varying chlorotic patches that may pass through the leaf veins.

Citrus Health Response Program. The goals of the Citrus Health Response Program (CHRP) are as follows:

- Sustain the U.S. citrus industry,
- Maintain the grower's continued access to export markets, and
- Safeguard the other citrus growing states against a variety of citrus diseases and pests.

This is a collaborative effort involving growers, Federal and State regulatory personnel and researchers.

chlorosis. Yellowing of normally green tissue due to chlorophyll destruction in infected plants.

decontamination. Application of an approved chemical or other treatment to contaminated implements, material, or buildings to kill or deactivate a pathogen.

delimiting survey. Determination of the extent of an infestation (e.g., distribution, density) in an area where an exotic species has been detected.

detection survey. Survey conducted in a susceptible area **not** known to be infested with an exotic species to determine its presence.

evaluation survey. *See monitoring survey.*

fastidious phloem-limited. The quality of a pathogen that describes its ability to only survive within the phloem vascular system of a plant.

host. Species that provides food, shelter or reproductive requirements for another organism.

identification authority. Authority to confirm the presence of a particular pest organism, issued by the PPQ National Identification Services to diagnosticians that have demonstrated proficiency in identifying.

infection. Establishment of a parasite on or within a host plant.

MDL. PPQ–National Identification Services–Molecular Diagnostics Laboratory, Beltsville, Maryland.

monitoring survey. Survey conducted at a site where a disease was found and where an eradication program is being performed. Also known as an evaluation survey.

necrosis. Dead or discolored plant tissue.

NPAG. New Pest Advisory Group.

NPB. The National Plant Board is an organization of the plant pest regulatory agencies of each of the states and Commonwealth of Puerto Rico.

NPGBL. National Plant Germplasm and Biotechnology Laboratory, Beltsville, Maryland.

pathogen. Any organism that can incite a disease.

PCR. Polymerase Chain Reaction, a laboratory technique that amplifies DNA sequences in order to determine if a host is infected with a known pathogen.

PPQ. Plant Protection and Quarantine.

potentially actionable suspect sample. Also know as PASS, a presumptive positive sample diagnosed or identified by provisionally approved laboratory or diagnostician with identification authority that would require confirmatory testing by an official APHIS Laboratory due to the nature of the plant sampled and the necessity for Federal confirmation.

presumptive positive. Such a result may require confirmatory testing if the sample is a PASS sample.

rapid delimiting survey. Survey method after a first detection in an area, deploy quickly with teams in designated increments (in radii or linear) away from the known focus detection.

rutaceous. Members of the plant family Rutaceae.

sentinel survey. Survey method that designates particular plants, of susceptible species or varieties, for repeated visits over a predetermined time period.

symptom. External and internal reactions or alterations of a plant as the result of a disease.

targeted survey. Choosing an area, usually residential, to concentrate surveys based on known pathway information from source countries with zip code based demographic information, also known as a hot zone survey.

traceback. To investigate the origin of infested plants through intermediate steps in commercial distribution channels to the origin.

trace forward. To investigate where infected plants may have been distributed from a source through steps in commercial distribution channels.

vector. Carrier of an infectious agent; capable of transmitting infection from one host to another; especially the animal that transfers an infectious agent from one host to another, usually an arthropod.

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A

Citrus Greening Disease

Appendix A

Resources

Diagnostic Tools and Equipment

FastPrep® FP120
Qbiogene
Corporate Headquarters
15 Morgan
Irvine, CA 92618-2005
Email: info@qbiogene.com
http://www.qbiogene.com/index.shtml

SmartCycler®
Cepheid
Sunnyvale, CA
http://www.cepheid.com

Biospec Products, Inc.
P. O. Box 788
Bartlesville, OK 74005

Invitrogen Corporation
1600 Faraday Avenue
P. O. Box 6482
Carlsbad, California 92008
Telephone: (760) 603-7200
Fax: (760) 602-6500
http://www.invitrogen.com

DNeasy® Plant Mini Kit
Qiagen
Valencia, CA
http://www1.qiagen.com/

Integrated DNA Technologies
http://www.IDTDNA.com

USDA-APHIS-PPQ-CPHST
National Plant Germplasm and Biotechnology Laboratory
BARC-East, Bldg 580
Powder Mill Rd
Beltsville, MD 20705
Telephone: 301-504-7100
Fax: 301-504-8539

Mary E. Palm
USDA–APHIS–PPQ–National Identification Services
Molecular Diagnostics Laboratory
Bldg. 580
Powder Mill Road
Beltsville, MD 20705
Telephone: 301-504-7154 or 504-5700 x 327
F: 301-504-6124
mary.palm@aphis.usda.gov

BioVentures
http://www.bioventures.com)

Environmental Compliance

Susan J. O'Toole
USDA–APHIS–PPQ
Environmental Services
Pesticide Labeling Issues
4700 River Road
Riverdale, MD 20737
Telephone: (301) 734-5861

Robert Baca
USDA–APHIS–PPQ
Environmental Monitoring
4700 River Road
Riverdale, MD 20737
Telephone: (301) 734-7175

Insect Collection and Traps

Great Lakes IPM, Inc.
10220 Church Road
Vestaburg, MI 48891
Telephone: 1-800-235-0285
Fax:(989) 268-5311
[http://www.greatlakesipm.com/]

BioQuip Products, Inc.
2321 Gladwick Street
Rancho Dominguez, CA 90220
Telephone: (310) 667-8800
[http://www.bioquip.com/]

B

Citrus Greening Disease

Appendix B

Forms

Contents

Introduction

Appendix B Forms provides examples of PPQ forms, and instructions for completing and distributing the forms.

PPQ 391 Specimens For Determination

This report is authorized by law (7 U.S.C. 147a). While you are not required to respond your cooperation is needed to make an accurate record of plant pest conditions. *See reverse for additional OMB information.*

FORM APPROVED
OMB NO. 0579-0010

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE

SPECIMENS FOR DETERMINATION

Instructions: Type or print information requested. Press hard and print legibly when handwritten. Item 1 - assign number for each collection beginning with year, followed by collector's initials and collector's number. Example (collector, John J. Dingle): 83-JJD-001.
Pest Data Section – Complete Items 14, 15 and 16 or 19 or 20 and 21 as applicable. Complete Items 17 and 18 if a trap was used.

FOR IIBIII USE
LOT NO.
PRIORITY

1. COLLECTION NUMBER

2. DATE — MO | DA | YR

3. SUBMITTING AGENCY
☐ State Cooperator ☐ PPQ ☐ Other _____

SENDER AND ORIGIN

4. NAME OF SENDER

6. ADDRESS OF SENDER

ZIP

INTERCEPTION SITE

5. TYPE OF PROPERTY *(Farm, Feedmill, Nursery, etc.)*

7. NAME AND ADDRESS OF PROPERTY OR OWNER

COUNTRY/ COUNTY

PURPOSE

8. REASON FOR IDENTIFICATION *("x" ALL Applicable Items)*

A. ☐ Biological Control (Target Pest Name)
B. ☐ Damaging Crops/Plants
C. ☐ Suspected Pest of Regulatory Concern *(Explain in* REMARKS*)*
D. ☐ Stored Product Pest
E. ☐ Livestock, Domestic Animal Pest
F. ☐ Possible Immigrant *(Explain in* REMARKS*)*
G. ☐ Survey *(Explain in* REMARKS*)*
H. ☐ Other *(Explain in* REMARKS*)*

9. IF PROMPT OR URGENT IDENTIFICATION IS REQUESTED, PLEASE PROVIDE A BRIEF EXPLANATION UNDER "REMARKS".

HOST DATA

10. HOST INFORMATION
NAME OF HOST *(Scientific name when possible)*

11. QUANTITY OF HOST
NUMBER OF ACRES/PLANTS
PLANTS AFFECTED *(Insert figure and indicate* ☐ Number ☐ Percent*):*

12. PLANT DISTRIBUTION
☐ LIMITED
☐ SCATTERED
☐ WIDESPREAD

13. PLANT PARTS AFFECTED
☐ Leaves, Upper Surface ☐ Trunk/Bark ☐ Bulbs, Tubers, Corms ☐ Seeds
☐ Leaves, Lower Surface ☐ Branches ☐ Buds
☐ Petiole ☐ Growing Tips ☐ Flowers
☐ Stem ☐ Roots ☐ Fruits or Nuts

PEST DATA

14. PEST DISTRIBUTION
☐ FEW
☐ COMMON
☐ ABUNDANT
☐ EXTREME

15. ☐ INSECTS ☐ NEMATODES ☐ MOLLUSKS

NUMBER SUBMITTED	LARVAE	PUPAE	ADULTS	CAST SKINS	EGGS	NYMPHS	JUVS.	CYSTS
ALIVE								
DEAD								

16. SAMPLING METHOD

17. TYPE OF TRAP AND LURE

18. TRAP NUMBER

19. PLANT PATHOLOGY – PLANT SYMPTOMS *("X" one and describe symptoms)*
☐ ISOLATED ☐ GENERAL

20. WEED DENSITY
☐ FEW ☐ SPOTTY ☐ GENERAL

21. WEED GROWTH STAGE
☐ SEEDLING ☐ VEGETATIVE ☐ FLOWERING/FRUITING ☐ MATURE

22. REMARKS

23. TENTATIVE DETERMINATION

24. DETERMINATION AND NOTES *(Not for Field Use)*

SIGNATURE

DATE

FOR IIBIII USE
DATE RECEIVED
NO.
LABEL
SORTED
PREPARED
DATE ACCEPTED
RR

PPQ FORM 391 *Previous editions are obsolete.*
(AUG 02)

This is a 6-Part form. Copies must be disseminated as follows:
☐ PART 1 – PPQ ☐ PART 2 – RETURN TO SUBMITTER AFTER IDENTIFICATION ☐ PART 3 – IIBIII OR FINAL IDENTIFIER
☐ PART 4 – INTERMEDIATE IDENTIFIER ☐ PART 5 – INTERMEDIATE IDENTIFIER ☐ PART 6 – RETAINED BY SUBMITTER

FIGURE B-1 Example of PPQ 391 Specimens For Determination [side 1]

OMB Information
According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0579-0010. The time required to complete this information collection is estimated to average .25 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

Instructions
Use PPQ Form 391, Specimens for Determination, for domestic collections (warehouse inspections, local and individual collecting, special survey programs, export certification).

BLOCK	INSTRUCTIONS
1	1. Assign a number for each collection beginning the year, followed by the collector's initials and collector's number **EXAMPLE** In 2001, Brian K. Long collected his first specimen for determination of the year. His first collection number is 01-BLK-001 2. Enter the collection number
2	Enter date
3	Check block to indicate Agency submitting specimens for identification
4	Enter name of sender
5	Enter type of property specimen obtained from (farm, nursery, feedmill, etc.)
6	Enter address
7	Enter name and address of property owner
8A-8L	Check all appropriate blocks
9	Leave Blank
10	Enter scientific name of host, if possible
11	Enter quantity of host and plants affected
12	Check block to indicate distribution of plant
13	Check appropriate blocks to indicate plant parts affected
14	Check block to indicate pest distribution
15	• Check appropriate block to indicate type of specimen • Enter number specimens submitted under appropriate column
16	Enter sampling method
17	Enter type of trap and lure
18	Enter trap number
19	Enter X in block to indicate isolated or general plant symptoms
20	Enter X in appropriate block for weed density
21	Enter X in appropriate block for weed growth stage
22	Provide a brief explanation if Prompt or URGENT identification is requested
23	Enter a tentative determination if you made one
24	Leave blank

Distribution of PPQ Form 391
Distribute PPQ Form 391 as follows:
1. Send Original along with the sample to your Area Identifier.
2. Retain and file a copy for your records.

FIGURE B-2 Example Of PPQ 391 Specimens For Determination [side 2]

Instructions

Block 1—Enter and label the following identification:

- Sample ID number, including 2-letter state code,
- Survey ID (in parenthesis), and
- State's own sample accession number.

Block 22—Enter the following information:

- Name of the office or diagnostic laboratory forwarding the sample;
- Name, e-mail address, and phone number of the contact; and
- Date forwarded to PPQ–National Identification Services–Molecular Diagnostics Laboratory (MDL).

Block 23—Enter the preliminary diagnosis (high or medium suspect CG). Inspectors must provide all relevant collection information with samples. This information should be communicated within a State and with the regional office program contact. If a sample tracking database is available at the time of the detection, please enter collection information in the system as soon as possible.

Determination Section—Diagnostic screening laboratories must write their determinations for each sample on the PPQ Form 391 that came with the sample. Include the name and phone number of the responsible diagnostician. Keep a copy for your records. Follow the same sample packaging instructions as above. Add the following additional information to the PPQ Form 391:

- Specific plant part that tested positive (i.e., the midrib);
- Method of PCR and method of DNA extraction (if other than APHIS authorized protocols were used); and
- Labeled images of the PCR test; use email to send images to Mary Palm while the plant material is en route and prior to testing.

Address

Mary E. Palm
USDA–APHIS–PPQ–National Identification Services
Molecular Diagnostics Laboratory
Bldg. 580, BARC East
Powder Mill Road
Beltsville, MD 20705
T: 301-504-7154 or 504-5700 ext 327
F: 301-504-6124
Email: mary.palm@aphis.usda.gov

- Images of associated symptomatic foliage and fruit from samples rated as high suspect for CG

PPQ 523 Emergency Action Notification

According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information is 0579-0102. The time required to complete this information collection is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

FORM APPROVED - OMB NO. 0579-0102

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
PLANT PROTECTION AND QUARANTINE

EMERGENCY ACTION NOTIFICATION

SERIAL NO.

1. PPQ LOCATION | 2. DATE ISSUED

3. NAME AND QUANTITY OF ARTICLE(S)

4. LOCATION OF ARTICLES

5. DESTINATION OF ARTICLES

6. SHIPPER

7. NAME OF CARRIER

8. SHIPMENT ID NO.(S)

9. OWNER/CONSIGNEE OF ARTICLES

Name:

Address:

PHONE NO. | FAX NO.

SS NO. | TAX ID NO.

10. PORT OF LADING | 11. DATE OF ARRIVAL

12. ID OF PEST(S), NOXIOUS WEEDS, OR ARTICLE(S)

12a. PEST ID NO. | 12b. DATE INTERCEPTED

13. COUNTRY OF ORIGIN | 14. GROWER NO.

15. FOREIGN CERTIFICATE NO.

15a. PLACE ISSUED | 15b. DATE

Under Sections 411, 412, and 414 of the Plant Protection Act (7 USC 7711, 7712, and 7714) and Sections 10404 through 10407 of the Animal Health Protection Act (7 USC 8303 through 8306), you are hereby notified, as owner or agent of the owner of said carrier, premises, and/or articles, to apply remedial measures for the pest(s), noxious weeds, and or article(s) specified in Item 12, in a manner satisfactory to and under the supervision of an Agriculture Officer. Remedial measures shall be in accordance with the action specified in Item 16 and shall be completed within the time specified in Item 17.

AFTER RECEIPT OF THIS NOTIFICATION, ARTICLES AND/OR CARRIERS HEREIN DESIGNATED MUST NOT BE MOVED EXCEPT AS DIRECTED BY AN AGRICULTURE OFFICER. THE LOCAL OFFICER MAY BE CONTACTED AT:

16. ACTION REQUIRED

☐ TREATMENT:

☐ RE-EXPORTATION:

☐ DESTRUCTION:

☐ OTHER:

Should the owner or owner's agent fail to comply with this order within the time specified below, USDA is authorized to recover from the owner or agent cost of any care, handling, application of remedial measures, disposal, or other action incurred in connection with the remedial action, destruction, or removal.

17. AFTER RECEIPT OF THIS NOTIFICATION COMPLETE SPECIFIED ACTION WITHIN (Specify No. Hours or No. Days):

18. SIGNATURE OF OFFICER:

ACKNOWLEDGMENT OF RECEIPT OF EMERGENCY ACTION NOTIFICATION

I hereby acknowledge receipt of the foregoing notification.

SIGNATURE AND TITLE: | DATE AND TIME:

19. REVOCATION OF NOTIFICATION

ACTION TAKEN:

SIGNATURE OF OFFICER: | DATE:

PPQ FORM 523 (JULY 2002) Previous editions are obsolete.

FIGURE B-3 Example of PPQ 523 [http://www.aphis.usda.gov/library/forms/pdf/ppq523.pdf]

Instructions

Use the following instructions to complete EAN PPQ Form 523.

Block 1—Enter the name or location of the nearest PPQ office.

Block 3—Enter the host scientific name and cultivar.

Block 4—Enter the following:

- Property address,
- Nursery,
- Grove number or name, and
- Other information indicating the location of the plant material held.

Block 6—Enter the plant material source, if known.

Block 7—Leave blank unless that information is known.

Block 8—Leave blank unless that information is known.

Block 12—Use Block 12 to place plant material on a property on hold. Enter *Candidatus* Liberibacter asiaticus or the correct name of another form of CG. The authority under which actions are taken is The Plant Protection Act of 2000 (Statute 7 USC 7701-7758).

Block 15—Indicate action required with suggested text as follows:

> All host plants of the *Candidatus* Liberibacter asiaticus pathogen and psyllid hosts are prohibited from movement from the property pending further notification by USDA–APHIS–PPQ or the state department of agriculture. No other disease host material, or host material of the insect *Diaphorina citri*, may leave the property until further evaluations can be made. After further investigations are conducted, the listed plants and other host material, if a positive detection is confirmed on the property, will be treated/destroyed under supervision, with approved methods in accordance with USDA and state policies. Any additional hosts the insect vector on the property are subject to Federal and state quarantine requirements prior to movement from the property.

C

Citrus Greening Disease

Appendix C

Symptoms On Citrus

Contents

Foliar Feeding by African Citrus Psyllid

FIGURE C-4 Pit galls caused by African citrus psyllid (*Trioza erytreae*) on upper surfaces (left) and lower surfaces (right) of citrus leaves [Images courtesy of Food and Agriculture Organization of the United Nations (FAO)]

FIGURE C-5 Pit galls caused by African citrus psyllid (*Trioza erytreae*) on citrus leaves [Left image courtesy of Peter Stephen, Citrus Research International, South Africa; right image courtesy of FAO]

Symptoms of Citrus Greening Disease on Trees and Foliage

FIGURE C-6 Yellow shoots on citrus caused by citrus greening disease [Image courtesy of T. R. Gottwald and S. M. Garnsey]

FIGURE C-7 Leaf mottling on citrus caused by citrus greening disease [Image courtesy of J.M. Bové and M. Garnier; reprinted from Timmer *et al.,* (2000)]

FIGURE C-8 *Left:* Vein yellowing and mottling of citrus caused by citrus greening disease [Image courtesy of Florida Department of Agriculture and Consumer Services] *Right:* Vein yellowing of citrus caused by citrus greening disease (Top); Healthy citrus leaf on bottom

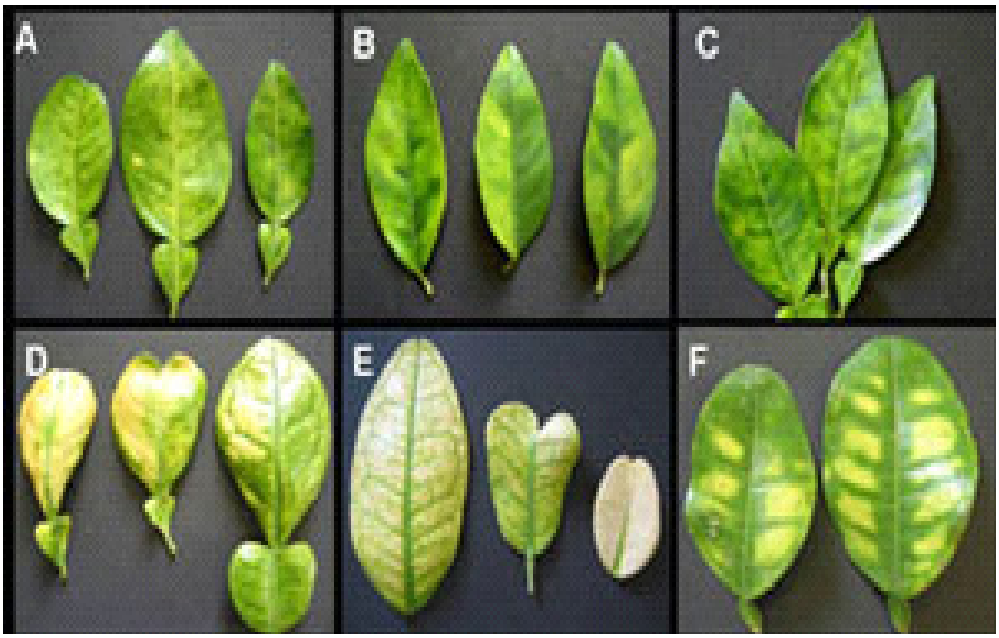

FIGURE C-9 Yellow mottling of leaves, caused by citrus greening disease on various citrus in Florida. A: Sour orange (*Citrus aurantium*); B: Lime (*Citrus aurantifolia*); D: Unknown (*Citrus* sp.); C, E, and F: Pummelo (*C. maxima*) [Image courtesy of Xiaoan Sun, Florida Department of Agriculture and Consumer Services, Division of Plant Industry]

Symptoms of Citrus Greening Disease on Fruit

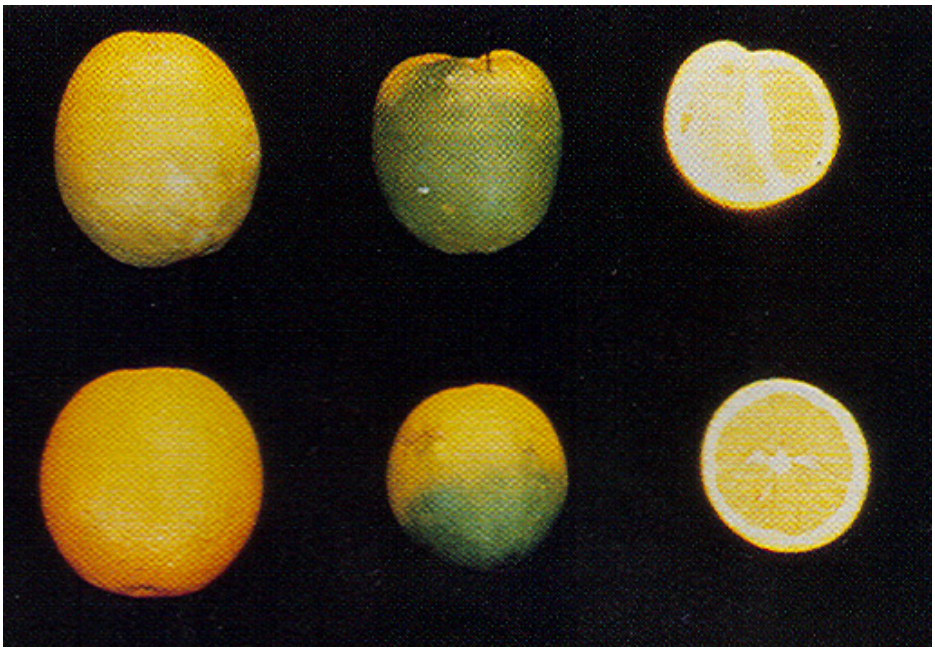

FIGURE C-10 Misshapen and greenish fruit of citrus caused by citrus greening disease [Healthy fruit is pictured in the lower left corner.] [Image courtesy of P. Broadbent and reprinted from Timmer *et al.,* (2000)]

FIGURE C-11 *Left:* Symptoms of citrus greening disease on mandarin orange fruit [Image courtesy T. R. Gottwald and S.M. Garnsey] *Right:* Cross section of fruit with citrus greening symptoms [Image courtesy of Xiaoan Sun, Florida Department of Agriculture and Consumer Services, Division of Plant Industry]

Similar Symptoms

FIGURE C-12 Yellowing of citrus leaves caused by lack of zinc

D

Citrus Greening Disease

Appendix D

Identification of Psyllids

Contents

Collection and Preparation of Specimens

If the vectors of citrus greening disease (CG) are present and carry the pathogen, then the area is at risk for the disease.

Psyllid identification is important as a possible indicator of disease presence in a geographical area. Currently, methods exist to detect the presence of the pathogen within the vector itself. Collecting and analyzing vectors from an area can be an important method for learning about CG epidemiology.

Collect as many specimens, adults and nymphs as possible for identification. Do **not** mix samples. Be sure to separate insects into vials by tree or location.

PPQ Form 391

Prepare **PPQ 391 Specimens For Determination on page B-2** and **PPQ 523 Emergency Action Notification on page B-5** and include the following information:

- Date of collection;
- Sample number from predetermined numbering;
- Collector's name and agency;
- Full address including county;
- Type of property (i.e., residential, nursery, commercial grove, feral or abandoned grove);
- Grower's field ID numbers, if appropriate;
- GPS coordinates of the host plant and property;
- Host species and cultivar;
- Observations of the number of trees exhibiting symptoms; and
- General conditions or any other relevant information.

Preparation of Specimens

Prepare specimens according to the following protocols:

- Gather nymphs/adults from the host plant and place in the same vial;
- Label the vial with a sample number, date, locale, etc.;
- Preserve the insects in 70% alcohol, or in 95% alcohol for PCR analysis;
- Use Fed-Ex® to ship vials in a well-padded box; include absorbent materials in case of vial breakage or leaks. Place box inside a resealable bag; and
- Include a completed PPQ Form 523, with the submitter's e-mail address on the form. **See PPQ 523 Emergency Action Notification on page B-5** for more information.

Submit specimens to your state or cooperating university entomologist for screening. If the psyllid vectors of CG are **not** known to occur in the state, and suspect detection is made, fill out a separate PPQ Form 391 marked Urgent and forward to the Leader, Taxonomic Services Unit, Beltsville, MD. For instructions on completing PPQ Form 391, see **PPQ 391 Specimens For Determination on page B-2**.

Asian Citrus Psyllid

Adults of the Asian citrus psyllid, *Diaphorina citri* Kawayama 1908 (=*Eupharalus citr*i) can be separated from most other *Diaphorina* species on citrus and citrus relatives by distinctive wing patterning (**Table D-13**, **Table D-14** and **Table D-15**). Halbert and Manjunath (2004) provided a review of the biology and taxonomic references for the other species. They also listed several other psyllid genera typically found on citrus.

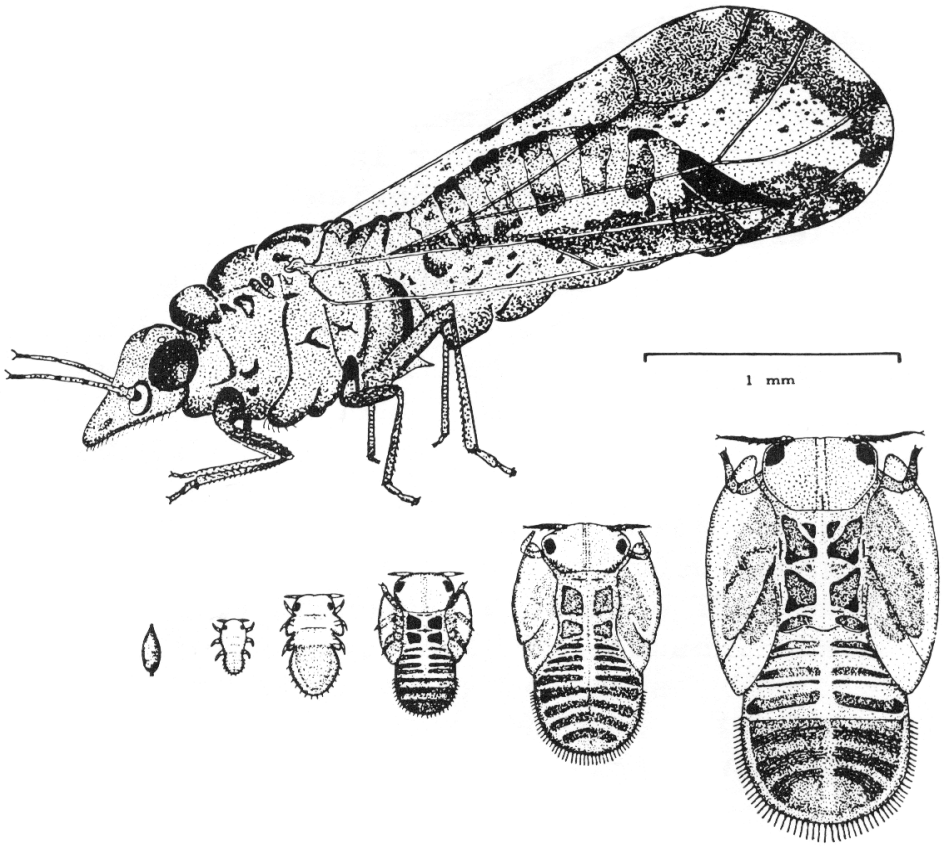

FIGURE D-13 Adult and nymphal instars of Asian citrus psyllid, *Diaphorina citri* Kuwayama; Illustration from Catling (1970)

FIGURE D-14 Asian citrus psyllid (*Diaphorina citri* Kuwayama) in typical feeding position with raised abdomen [Image courtesy of David Hall, USDA–ARS, Ft. Pierce, FL]

FIGURE D-15 Asian citrus psyllid (*Diaphorina citri* Kuwayama) nymphal instars [Image courtesy of David Hall, USDA–ARS, Ft. Pierce, FL]

African Citrus Psyllid

The African citrus psyllid (*Trioza erytreae* [del Guercio] 1918) is difficult to separate morphologically from ten other species in the genus, but can be separated by host preferences (Halbert and Manjunath, 2004) (**Table D-16**, **Table D-17** and **Table D-18**).

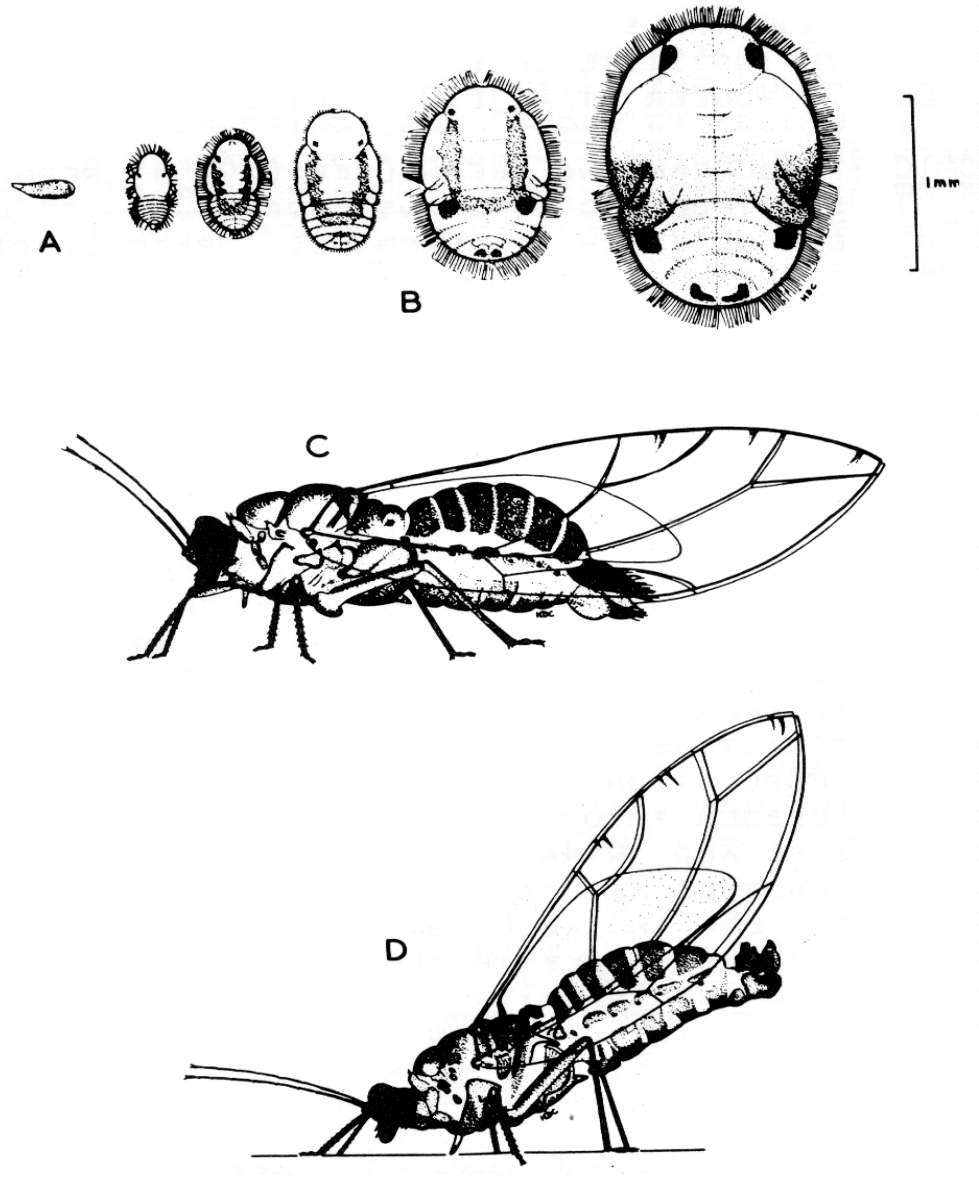

FIGURE D-16 Egg (A), nymphal instars (B), and adults (C and D) of African citrus psyllid *Trioza erytreae* (del Guercio) [Illustration from Catling and Annecke (1968)]

FIGURE D-17 African citrus psyllid *Trioza erytreae* (Del Guercio) with eggs [Image courtesy of S. P. Van Vuuren, Citrus Research International, South Africa]

FIGURE D-18 African citrus psyllid *Trioza erytreae* Del Guercio nymph [Image courtesy of Peter Stephen, Citrus Research International, South Africa]

Appendix E

DNA Extraction and PCR Detection in Citrus

Work Instructions

March 23, 2007. Plant sample extraction for use in citrus greening or huanglongbing molecular diagnostic assays.

March 26, 2007. Real-time PCR for diagnostic detection of citrus greening or huanglongbing from psyllid samples.

March 26, 2007. Real-time PCR for diagnostic detection of citrus greening or huanglongbing from plant samples.

March 26, 2007. Psyllid sample extraction for use in citrus greening or huanglongbing molecular diagnostic assays.

This work instruction provides information on DNA extraction from suspect plant samples collected from the field for the purpose of Citrus Greening or HLB (Huanglongbing) molecular detection by PCR. The extraction uses a commercial kit containing a spin column.

Related Work Instruction

WI-B-T-D-2 Real-time PCR for Diagnostic Detection of Citrus Greening or HLB (Huanglongbing) from Plant Samples

I. Introduction

Citrus Huanglongbing (HLB), ex citrus greening, is one of the most serious insect-vectored pathogens of citrus. The disease is wide spread in Asia, the Indian Subcontinent and Ocean, southern Africa and Brazil. In August 2005, HLB was detected in south Miami-Dade County, Florida, and since then in several additional counties in Florida. The pathogen is a fastidious prokaryote, in the ά- subdivision of the Proteobacteria and has not yet been cultured on artificial media. The bacterium resides in the sieve tube elements of infected plants and in infected insects. HLB exists in nature in three forms that differ by a combination of environmental conditions and insect vectors. HLB caused by *Candidatus* Liberibacter asiaticus (Las), a heat-tolerant form found in Asia, is vectored by *Diaphorina citri*. HLB caused by *Ca.* L. africanus (Laf), a heat-sensitive form found in southern Africa, is vectored by *Trioza erytreae*. HLB caused by *Ca.* L. americanus (Lam), a heat-tolerant form found in Brazil, is vectored by *D. citri*. Conventional PCR methods using specific primers that amplify 16S rDNA sequences have been used to detect Las and Laf (Jagoueix et al., 1996; Tian et al, 1996), but require digestion of the PCR products with *Xba1* to distinguish between them. A new primer set, developed in 1999, based on β-operon protein genes of the, can be used to detect and differentiate Las from Laf directly by amplicon size (Hocquellet et al., 1999).

Although conventional PCR assays exist, rapid detection methodologies necessary for a regulatory response and effective management of HLB were lacking. It has been difficult to consistently detect the liberibacters through the use of biological assays, the presence of fluorescent substances, light or electron microscopy, or ELISA. This is presumably because of the low concentration and uneven distribution of the pathogens in host plants and vector insects. In addition, the non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutrient deficiencies or other citrus diseases. Molecular approaches have successfully been used to detect and differentiate liberibacter species of HLB.

I. Equipment, Material and Reagents

A. Equipment

1. Biological Safety Cabinet, Class II, Type A/3B (now referred to as II, A2)
2. FastPrep FP-120 or -24 instrument (Qbiogene, Inc) or Mini-bead Beater (BioSpec)
3. Balance, capable of weighing 20-100 mg
4. Thermomixer (Eppendorf #5350), or waterbath, capable of 56°C
5. Microcentrifuge, bench-top, capable of ≥10,000 rpm
6. Vortex
7. Freezer, -20, (non-frost free)
8. Dedicated, annually-calibrated pipettors (P10, P50, P200)

B. Materials

1. Qiagen® DNeasy Plant Mini Kit, #69104 (50 tests) or #69106 (250 tests)
2. Sterile filter (barrier) pipette tips (P10, P50, P200)
3. Gloves (any vendor)
4. Weigh boats (any vendor)
5. Razor blades, single-use (any vendor)
6. Microcentrifuge tubes, 1.7 ml (pre-sterilized, certified DNase & RNase free, any vendor)
7. Paper towels, absorbent (any vendor)
8. Disposable, absorbent bench underpads (any vendor)
9. Ice

10. Microcentrifuge tube openers (any vendor)

11. For Fastprep instrument: Lysing Matrix A tubes (Qbiogene #6910-050)

12. For Mini bead Beater instrument: Microcentrifuge tubes, 2 ml, screw cap tube, o-ring seals sterile, RNase, DNase, DNA, and pyrogen free, capable of withstanding bead beating (USA Scientific, 1420-9710 or 1420-8710) and Chrome-steel beads, 6.35 mm, BioSpec part no. 11079635c

13. Tube openers (any vendor)

Notes:

All snap closure microcentrifuge tubes are to be opened using a decontaminated tube opener. Tube openers are decontaminated by soaking 2 hours in 10% bleach solution, followed by two rinses in water and distilled water to remove bleach residue.

If you have received packages from different states on the same day, process the samples of the first package completely (sections III and IV), then decontaminated the work area and change out all disposable prior to opening and processing the next package(s).

To minimize the potential for general contamination the DNA extraction area should be separated from the PCR work.

To avoid cross contamination of samples, use a new razor blade, new gloves, new lab mats, and new weighing boats/paper with each sample.

III. Sample Preparation for extraction

Sample package opening, sampling and weighing must be conducted in a Biological Safety Cabinet Class II, Type A/B3.

1. Select four twigs of this year's growth with Huanglongbing (*HLB*) or *HLB*-like symptoms collected from the upper canopy of a suspect tree.

2. Remove the third through the fifth leaves from the top of each twig and place in an new weigh boat.

3. Remove the midribs with petioles from the 12 selected leaves using a new razor blade and cut them into pieces of about 5 mm in length in a Petri dish or new weigh boat. The remainder of the midrib pieces can be stored at -20ºC for use in the future.

IV. DNA Extraction

A. Disruption using Qbiogene FastPrep instrument and Qiagen DNeasy® Plant Mini kit *(*Modified for sample weight and buffer volumes)

1. Weigh out 200 mg (a modified weight) of chopped midribs and petioles and put into a Qbiogene Lysing Matrix A tube. (Each 2.0 ml tube contains garnet matrix and one 1/4 inch ceramic sphere and can be ordered in larger quantities such as 100 and 500.)

2. Add 800 µl (a modified volume) of the Qiagen DNeasy® Plant Mini extraction kit AP1 buffer and 8 µl (a modified volume) of RNase A (100 mg/ml) into a sample tube.

   Note: These are modified volumes because we start with more plant tissue than recommended by the Manufacturer in step 4 above.

3. Homogenize plant tissues at a speed 6.0 for 40 second.

4. Centrifuge sample for 10 seconds at ≥10,000 rpm to remove tissue from the cap prior to adding extraction buffer.

5. Proceed to step B.5 below to complete the extraction.

B. Disruption using the BioSpec BeadBeater and Qiagen DNeasy® Plant Mini kit *(*Modified for sample weight and buffer volumes)

1. Weigh out 200 mg (a modified weight) of chopped midribs and petioles and put into a 2ml screw capped tube with o-ring seals that has straight edges and a sharp conical (pointed) bottom.

   Note: We do not recommend using the microplate format with the BioSpec-96 bead beader. Please only use screw cap tubes to prevent cross contamination.

2. Add 2, 6.35 mm, chrome-steel beads.

3. Add 800 µl of the Qiagen DNeasy® Plant Mini extraction kit AP1 buffer and 8 µl of RNase A (100 mg/ml) into the sample tube. (These are modified volumes because we used more plant tissue in step 4 above).

   Note: After speaking with the BioSpec technical representatives, freezing the tissue with liquid nitrogen is not a necessary step in the DNA extraction when using the Qiagen extraction kits. However, you must add the liquid buffer prior to homogenizing with the chrome steel beads or you will shatter the tubes.

4. Homogenize samples according to the type of MiniBeadBeater (MBB) unit available, for a total of three minutes. For the MBB with single tube capacity use the settings of 48 (4800 rpm) and a time of 18 (180 seconds / 3 minutes). For the MBB-8, set the speed at Homogenize and run the cycle for a total "on" time of three minutes.

   NOTE: With all MiniBeadBeater systems, test the system settings out with mock samples prior to use to avoid tube shatter when processing samples.

5. Using a shaking thermomixer, incubate cellular lysate at 65ºC for 10 min with 300-rpm agitation.

6. Add 260 µl (a modified volume) of Buffer AP2 to the lysate, vortex briefly and incubate on ice for 5 min.

7. Centrifuge at 14,000 rpm for 10 min. (a modified time). Transfer the lysate to a DNeasy® QIAshredder Mini Spin Column (lilac colored column [included in the kit]) in a 2 ml collection tube and centrifuge for 2 min at 14,000 rpm.

8. Transfer the flow-through to a new tube without disturbing the pellet. Typically about 500 µl of lysate can be recovered.

9. Measure the volume and add 1.5 volumes of Buffer AP3/E to the lysate and mix by pipetting.

10. Apply 650 µl of the mixture, including any precipitate, to the DNeasy® Mini Spin Column sitting in a new 2 ml collection tube. Centrifuge at ≥ 8000 rpm for 1 min. (Discard flow-through.)

11. Repeat Step 10 with the remaining portion of the mixture. Discard flow-through and collection tube.

12. Place the column in a new 2 ml collection tube. Add 500 µl of Buffer AW to the column and centrifuge at ≥8000 rpm for 1 min. Discard flow-through and reuse the collection tube.

13. Do a second wash by adding 500 µl Buffer AW to the spin column and centrifuge for 2 min at 14,000 rpm to dry the membrane.

14. Transfer the column to a new 1.5 ml microcentrifuge tube and pipet 100 µl of pre-warmed (65ºC) Buffer AE onto the column membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at ≥ 8000 rpm to collect DNA elution. (Do not allow the column to dry.)

15. Repeat step 14 once, to collect a total of 200 µl of DNA.

16. Extracts of total genomic DNA can be stored at 4º C for immediate use only or at -20ºC for use in the future.

Document Revision History

Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	3-23-07	To baseline the work instruction.

Approved: Signature on File with Original Document
Date: 3-23-2007

Approved By: Renee M. DeVries, NPGBL Quality Manager
Signature:

Approved By: Laurene Levy, NPGBL Technical Manager
Signature:

The purpose of this work instruction is to describe all activities concerning the **diagnostic detection** of Citrus Greening or HLB (Huanglongbing) caused by *Candidatus* Liberibacter asiaticus (Las) and L. americanus (Lam) from **psyllid samples**, by TaqMan quantitative PCR using 16s rDNA-based primers and probe (Li et al., 2006).

PCR testing is conducted on DNA (stored at -20°C) previously extracted (WI-B-T-1-16) from psyllids collected from the field. *This work instruction is not for use on plant DNA.*

Related Work Instruction

WI-B-T-1-16 *Psyllid Sample Extraction for use in Citrus Greening or HLB (Huanglongbing) Molecular Diagnostic Assays*

I. Introduction

Citrus Huanglongbing (HLB), ex citrus greening, is one of the most serious insect-vectored pathogens of citrus. The disease is wide spread in Asia, the Indian Subcontinent and Ocean, southern Africa and Brazil. In August 2005, HLB was detected in south Miami-Dade County, Florida, and since then in several additional counties in Florida. The pathogen is a fastidious prokaryote, in the ά- subdivision of the Proteobacteria and has not yet been cultured on artificial media. The bacterium resides in the sieve tube elements of infected plants and in infected insects. HLB exists in nature in three forms that differ by a combination of environmental conditions and insect vectors. HLB caused by *Candidatus* Liberibacter asiaticus (Las), a heat-tolerant form found in Asia, is vectored by *Diaphorina citri*. HLB caused by *Ca.* L. africanus (Laf), a heat-sensitive form found in southern Africa, is vectored by *Trioza erytreae*. HLB caused by *Ca.* L. americanus (Lam), a heat-tolerant form found in Brazil, is vectored by *D. citri*.

It has been difficult to consistently detect the liberibacters through the use of biological assays, the presence of fluorescent substances, light or electron microscopy, or ELISA. This is presumably because of the low concentration and uneven distribution of the pathogens in host plants and vector insects. In addition, the non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutrient deficiencies or other citrus diseases. Molecular approaches have successfully been used to detect and differentiate liberibacter species of HLB. Conventional PCR methods using specific primers that amplify 16S rDNA sequences have been used to detect Las and Laf (Jagoueix et al., 1996; Tian et al, 1996), but require digestion of the PCR products with *Xba1* to distinguish between them. A new primer set, developed in 1999, based on β-operon protein genes of the, can be used to detect and differentiate Las from Laf directly by amplicon size (Hocquellet et al., 1999).

Although conventional PCR assays exist, rapid detection methodologies necessary for a regulatory response and effective management of HLB were lacking. Real-time, quantitative PCR has gained acceptance due to its improved speed, sensitivity, reproducibility, robustness

and the reduced risk of carry-over contamination. A quantitative, multiplex, real-time, fluorogenic PCR (TaqMan®) assay was developed in 2005 using probe-primer sets specific to *Ca.* Liberibacter spp., and a plant cytochrome oxidase (COX)-based primer–probe set as a positive internal control (Li et al., 2006). This assay allows for a real-time field diagnosis in less than 1 hour using a portable SmartCycler® (Cepheid, Sunnyvale, CA) machine and DNA extracts. The real-time PCR assay differentiates HLB strains by using different forward primers for each HLB strain but the same reverse primer and probe.

Since detection of the pathogen in insects can be difficult due to varying pathogen concentration, it is important to note a negative test result indicates the pathogen was not detected but could be present at levels well below detection capabilities of the assay.

II. Equipment, Materials and Reagents

A. Equipment

1. Biological Safety Cabinet, Class II, Type A/B3 (new designation: Class II, A2)
2. PCR WorkStation
3. Smart Cycler® II – Cepheid
4. Cepheid Smart Cycler bench-top centrifuge (Cepheid # 900-0020))
5. Bench-top microcentrifuge capable of 14,000rpm
6. Vortex-Genie
7. Eppendorf Thermomixer (Brinkmann Instruments, Inc.)
8. Analytical Balance
9. Dedicated, annually-calibrated pipettors (P10, P50, P200)
10. Freezer (-20ºC, non-frost-free)

B. Materials

1. 1.7ml micro centrifuge tubes, clear and amber (pre-sterilized, certified DNase & RNase free, any vendor)
2. SmartCycler® tubes (25µl) (Cepheid #900-0022)
3. Sterile filter (barrier) pipette tips for the corresponding pipettes
4. Gloves
5. Disposable bench tissue paper (any vendor)
6. Absorbent disposable bench under pads (any vendor)
7. Microcentrifuge tube openers (any vendor)

C. Reagents

1. Molecular Grade (MG) water (any vendor)
2. TE buffer (any vendor)
3. Ethyl alcohol (200 proof)
4. Platinum® Taq DNA Polymerase (Invitrogen part # 10966-034)
   Note: Platinum *Taq* is supplied in a set with 10X PCR Buffer and 50mM $MgCl_2$

5. 10 mM dNTP mix PCR Grade (Invitrogen part #18427-013)
6. Primers and probes* (see tables for details) *Please note that probes take 7 to 14 business days to arrive once ordered.

All snap closure microtubes are to be opened using a decontaminated tube opener. Tube openers are decontaminated by soaking 2 hours in 10% bleach solution, followed by two rinses in water and diH_2O to remove bleach residue.

III. Real-time PCR Assay

A. Primers and Probes

Table 2. TaqMan primers

Primer Name	Primer Mix Name	Sequence 5' - 3' Synthesized by Integrated DNA Technologies, Inc.; Primer Purification = Standard Desalting	Target gene	Specific to
HLBas primer	**HLBas primer Mix**	TCG AGC GCG TAT GCA ATA CG	16S rDNA	Las
HLBr primer[1]		GCG TTA TCC CGT AGA AAA AGG TAG		
HLBam primer	**HLBam primer mix**	GAG CGA GTA CGC AAG TAC TAG	16S rDNA	Lam
HLBr primer[1]		GCG TTA TCC CGT AGA AAA AGG TAG		
WG primer	**WG[2] primer Mix**	GCT CTC AAA GAT CGG TTT GAC GG	WG* gene	Psyllids
WGr primer		GCT GCC ACG AAC GTT ACC TTC		

[1]The HLBas and HLBam primer mixes share one common primer, HLBr.
[2] WG refers to glycoprotein.
Note: the "r" in the primer name HLBr and WGr denotes the reverse primer.

Table 3. Probes for Real-time PCR (Synthesized by Integrated DNA Technologies, Inc.)

HLBp probe	56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ-1
WGp probe	5-TET/TTA CTG ACC ATC ACT CTG GAC GC/3BHQ-2

Note: Probes take 7 to 14 business days to arrive once ordered.

1. Tubes with lyophilized primers and probes (shown in Table 2 and 3) are centrifuged briefly (10-20 seconds at minimum 10,000 rpm) before opening to ensure that the lyophilized material is in the bottom of the tube.

2. Freezer stock solutions (100 µM) of the probes and primers
   Working inside a clean PCR workstation, primers and probes are re-hydrated to 100µM stock solution in 1xTE buffer and stored at - 20°C. Store freezer stocks of probes in amber-colored microcentrifuge tubes.

3. Working solutions of primer mixes
   Prepare a working solution of each of the **HLBas/HLBr, HLBam/HLBr** and **WG/WGr** primer mixes by adding 20 µl of the 100µM stock solution of the appropriate two primers of each pair mix to 960 µl 1xTE buffer (final concentration is 2 µM).

4. Working solutions of probes
   Prepare 1 µM solutions of each probe in molecular biology grade water or 1x TE buffer by diluting the 100 µM probe stock solution (add 10 µl of stock solution to 990 µl of 1xTE buffer, final concentration is1 µM). Store small aliquots (i.e.: 20 µl) of probes at -20ºC in 1.5 ml amber-colored microfuge tubes to protect the primer from degradation due to light exposure. Small aliquots, suitable for one-day use, of probe are desirable since probes are sensitive to frequent freeze/thaw cycles.

B. Preparing the Real-time PCR Master Mix

Master Mix preparation and aliquoting must be done in a clean PCR workstation.

1. Label two sets of Cepheid 25 µl tubes for samples and positive and negative controls.

2. Prepare two Real-time PCR master mixes (Table 4) inside a clean PCR workstation for the number of the samples plus additional (1 for every 10) samples, plus the 2 controls. Keep master mixes on ice once prepared and prior to aliquoting for individual reactions.

Table 4. Real-time PCR Master Mix

Reagents	1 reaction	10 reactions	Final concentration
Molec. Grade water	4.7 µl	47 µl	N/A
10x PCR buffer	2.5 µl	25 µl	1x
$MgCl_2$ (50mM)	3.0 µl	30 µl	6.0 mM
dNTPs (10 mM each) (Invitrogen)	0.6 µl	5.0 µl	0.24 mM
Platinum Taq (5U/µl) Invitrogen	0.2 µl	2.0 µl	1 Unit
HLB primer Mix* (2µM each)	3.0 µl	30 µl	240 nM
HLBp probe (1µM)	3.0 µl	30 µl	120 nM
WG primer Mix (2µM each)	3.0 µl	30 µl	240 nM
WGp probe (1µM)	3.0 µl	30 µl	120 nM
Total	23.0 µl	230 µl	N/A

* NOTE: "HLB Primer Mix" in table refers to either HLBas/HLBr or HLBam/HLBr. WG primer mix refers to WG/WGr.

3. Pipette up and down several times to thoroughly mix, then aliquot 23.0 µl of Master Mix into each 25 µl Cepheid tube.

C. Sample or Control addition to Real-time master mix

1. Take tubes containing master mix to the Cepheid cycler station and place all items on a new disposable lab mat.

2. Thaw frozen DNA samples briefly (several minutes) then vortex 5-10 seconds at speed setting of 7-10. Briefly spin the tubes 10-20 seconds at ≥ 10,000 rpm in a bench top microcentrifuge to settle the liquid at the bottom of the tube. Place in ice.
Add 2 µl of undiluted psyllid sample DNA to the corresponding Cepheid tube and close the cap. The total reaction volume is 25 µl.

2. Each diagnostic run of the real-time PCR must include an HLB-positive (Las or Lam as appropriate) psyllid DNA control and one negative control (negative template control, NTC). For the positive control reaction, add 2 µl of positive control DNA. Add 2 µl of MG water or TE buffer to the master mix for the negative control reaction. The total reaction volume is 25 µl.

> Note: Do not use 25 µl of master mix as your negative control. The negative control can indicate contamination of master mix reagents or water, or contamination introduced by the analyst during sample addition.

4. Close the tubes and spin them in the Cepheid centrifuge for 10 seconds. Make sure no bubbles remain on the sides of the flat diamond-shaped area of the Cepheid tube. Place the tubes in the SmartCycler® in the appropriate I-core modules.

D. Loading Smart Cycler® and starting the run

1. Turn on the SmartCycler® Block first, followed by the SmartCycler® software. If not done in this order the machine will give an error message.

2. Program Set-up:
   - Stage 1: Hold at 95ºC for 20 seconds with optics off.

   - Stage 2: repeat 40 times and 2-Temperature Cycle.
     -the first temperature cycle, set 95ºC for 1 second with optics off
     -the second temperature cycle, set 58ºC for 40 seconds with optics on.

Use a temperature ramping rate of 3 degrees per second.

3. To begin amplification: (instructions contained in the SmartCycler® manual)
   i. Select "Create Run"
   ii. Give the run a name (i.e.: date and protocol)
   iii. Select dye set (for this qPCR it is "FTTC25")
   iv. Select "add/Remove Sites" to select the protocol
   v. Select the sites with the samples and click on ► symbol to add sites to the right column, and click on OKAY.
   vi. Select "Start Run" found in the lower left-hand corner of the screen.

E. Assessment of the qPCR using the SmartCycler® software Version 2

Analyze data from the run as per the thermocycler Manufacturer's instructions

- If the HLB-positive psyllid control sample DNA has a FAM Ct = 0.00 or Ct 38-40, or your negative control has FAM Ct > 0.00, the entire run is invalid and all samples must be retested by real-time PCR.

- If the positive psyllid control has a WG internal control Tet Ct = 0.00, the entire run is invalid and all samples must be retested by real-time PCR.

- If a psyllid sample has a WG internal control Tet Ct = 0.00 or Ct 38-40, the specific sample must be retested. If a retest again produces the same result, the psyllid sample must be re-extracted as that DNA does not pass quality control.

- Las or Lam Positive samples: If a psyllid sample produced a FAM Ct value in the range of 0.00 <FAM Ct ≤ 32 in the real-time PCR the sample is determined to be positive for Las or Lam. (Note: The sample WG internal control for the real-time PCR must be acceptable.) Samples which test positive for HLB should be retested by real-time PCR to confirm the first run results. If a similar result is obtained, then the sample is determined to be positive. Also if this is a Potentially Actionable Suspect Sample (PASS), the sample must be forwarded for federal confirmation (see section F below).

- Inconclusive samples: If the psyllid sample produced a FAM Ct value in the range of 32 <FAM Ct <37, then the sample has tested inconclusive for Las or Lam and the real-time PCR needs to be rerun for this specific sample. If a similar result is obtained, the sample is determined to be inconclusive. If this is a Potentially Actionable Suspect Sample (PASS), the sample must be forwarded for federal confirmation (see section F below).

- Samples in which the pathogen (Las or Lam) was not be detected: If a psyllid sample produces a FAM Ct value of 0.00 or ≥37 then the result is "Las or Lam was not detected for this sample".

F. Samples that need to be forwarded for Federal confirmation

A Potentially Actionable Suspect Sample (PASS), in its simplest form, is a sample of a pest or pathogen of regulatory concern (including bacteria, viruses, nematodes, weeds, insects, mites, mollusks, etc) that have been presumptively identified by a laboratory without federal confirmatory authority.

The presumptive identification of a PASS by a non-approved laboratory would result in the sample(s)/specimen(s) or its biological extract (DNA, RNA, proteins, etc.) being forwarded to a designated Federal laboratory for confirmatory testing. Subsequent 'suspect' positive samples from within an APHIS-defined regulated area of the first PASS would not require federal confirmatory testing, but new finds outside of the defined area are considered a new PASS. Typically, a sample considered a PASS would also encompass any sample that involves unusual or unexpected circumstances, for example, a new host, new location, an atypical biology, or potential bio-terrorism act.

'Suspect' HLB-positive samples from within a regulated area(s). A 'suspect' HLB-positive sample from within a designated regulated area is not considered a PASS. That means this category of sample does not require Federal confirmatory testing *unless* it represents a new host or a yet unconfirmed Liberibacter species in which case it must be forwarded to the PPQ laboratories in Beltsville, MD. Positive identification of *Ca.* Liberibacter asiaticus or L. americanus from samples that are not PASS would be conducted at those laboratories certified by CPHST to perform PPQ validated diagnostic assays for HLB.

'Suspect' samples from outside a regulated areas. A 'suspect' HLB-positive sample from any citrus plant or citrus psyllid(s) from outside the regulated area is categorized as PASS. That means this category of sample must receive full Federal confirmatory testing. 'Suspect' sample(s) that qualify as a PASS that are determine in laboratories certified by CPHST to perform PPQ validated diagnostic assays for HLB will require Federal confirmatory testing that includes identification on the basis of testing of DNA isolated from citrus plant tissue or citrus psyllids. Confirmatory testing of all PASS for HLB is conducted at the USDA PPQ laboratories in Beltsville, MD.

Any sample that is submitted (overnight delivery) to NPGBL must be accompanied by a PPQ 391 Form (Sample Submission Form) and Ct values for each run (see example table below).

Please provide a table of your real-time PCR data with the sample(s):

Example of real-time PCR Ct table to send to NPGBL

Sample ID	PCR Run #	Dilution	HLB FAM Ct Value	WG TET Ct Value
HLB-*state code*	1	Undil	22.5	31.6

The NPGBL is authorized to receive a PASS sample for testing under APHIS permit # 90015 for domestic suspect select agent plant pathogens.

Please provide NPGBL with the tracking number and estimated delivery date by e-mail (laurene.levy@aphis.usda.gov, renee.m.devries@aphis.usda.gov), prior to sending material to NPGBL at the shipping address below:

Dr. Laurene Levy
USDA-APHIS-PPQ-CPHST-NPGBL
BARC-East, Bldg 580
Powder Mill Rd
Beltsville, MD 20705,
Phone 301-504-7100 and fax 301-504-8539

If the submitting diagnostic laboratory held back a portion of a PASS and the PASS is determined to be positive for HLB, a select agent (SA), there is an follow-up action required of the submitting laboratory under the Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331). The submitting diagnostic laboratory is required to notify the APHIS SA Program immediately and complete a SA form regarding the destruction or transfer to a SA registered entity. If you choose to destroy the remaining sample, it must take place within seven (7) days of results notification and a PPQ Office must witness the destruction of the sample on or before the 7-day period expires. See http://www.aphis.usda.gov/programs/ag_selectagent/index.html, or call 301.734.5960.

References

1. Jagoueix, S., Bove´, J.M., Garnier, M., 1996. PCR detection of the two Candidatus Liberobacter species associated with greening disease of citrus. Mol. Cell. Probes 10, 43– 50.

2. Tian, Y., Ke, S., Ke, C., 1996. Polymerase chain reaction for detection and quantification of Liberobacter asiaticum, the bacterium associated with huanglongbing (greening) of citrus in China. In: da Graca, J.V., Moreno, P., Yokomi, R.K. (Eds.), Proc. 13th Conf. Int. Org. Citrus Virol. (IOCV). University of California, Riverside, pp. 252–257

3. Li, Wenbin, Hartung, J.S., Levy, L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. J. Microbiol. Methods 66:104-115.

4. SmartCycler® II Operator Manual Copyright 1999-2002 by Cepheid.

Document Revision History

Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	3-21-2007	To baseline the Diagnostic work instruction
Revision	1	3-26-2007	Changed titles of this and the related work instruction (page 1). Added language to introduction regarding detection in insects. Removed conventional PCR related information. Added HLBam primer information (Table 2). Added language regarding primer and probe stock and working solutions. Added HLBam primers to Table 4. Added need for second set of tubes (III.B.1). Clarified that undiluted DNA is tested. Modified positive control information (III.C.3) to include HLB-Lam. Modified Assessment section (III.E), including Ct range for inconclusive samples and the need to retest positive and inconclusive samples.

Approved: Signature on File with Original Document

Date: 3-26-2007

Approved By: Renee M. DeVries, NPGBL Quality Manager
Approved By: Laurene Levy, NPGBL Technical Manager

The purpose of this work instruction is to describe all activities concerning the **diagnostic detection** of Citrus Greening or HLB (Huanglongbing) caused by *Ca*. L. asiaticus (Las) and L. americanus (Lam), from **plant samples,** by TaqMan quantitative PCR using 16s rDNA-based primers and probe (Li et al., 2006). **This work instruction replaces the previously approved WI-B-T-1-11 (rev. 3).**

PCR testing is conducted on DNA (stored at -20°C) previously extracted (WI-B-T-1-17) from leaves collected from the field. *This work instruction is not for use on psyllid DNA.*

Related Work Instructions

WI-B-T-1-17 *Plant Sample Extraction for use in Citrus Greening or HLB (Huanglongbing) Molecular Diagnostic Assays*

I. Introduction

Citrus Huanglongbing (HLB), ex citrus greening, is one of the most serious insect-vectored pathogens of citrus. The disease is wide spread in Asia, the Indian Subcontinent and Ocean, southern Africa and Brazil. In August 2005, HLB was detected in south Miami-Dade County, Florida, and since then in several additional counties in Florida. The pathogen is a fastidious prokaryote, in the ά- subdivision of the Proteobacteria and has not yet been cultured on artificial media. The bacterium resides in the sieve tube elements of infected plants and in infected insects. HLB exists in nature in three forms that differ by a combination of environmental conditions and insect vectors. HLB caused by *Candidatus* Liberibacter asiaticus (Las), a heat-tolerant form found in Asia, is vectored by *Diaphorina citri*. HLB caused by *Ca.* L. africanus (Laf), a heat-sensitive form found in southern Africa, is vectored by *Trioza erytreae*. HLB caused by *Ca.* L. americanus (Lam), a heat-tolerant form found in Brazil, is vectored by *D. citri*.

It has been difficult to consistently detect the liberibacters through the use of biological assays, the presence of fluorescent substances, light or electron microscopy, or ELISA. This is presumably because of the low concentration and uneven distribution of the pathogens in host plants and vector insects. In addition, the non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutrient deficiencies or other citrus diseases. Molecular approaches have successfully been used to detect and differentiate liberibacter species of HLB Conventional PCR methods using specific primers that amplify 16S rDNA sequences have been used to detect Las and Laf (Jagoueix et al., 1996; Tian et al, 1996), but require digestion of the PCR products with *Xba1* to distinguish between them. A new primer set, developed in 1999, based on β-operon protein genes of the, can be used to detect and differentiate Las from Laf directly by amplicon size (Hocquellet et al., 1999).

Although conventional PCR assays exist, rapid detection methodologies necessary for a regulatory response and effective management of HLB were lacking. Real-time, quantitative

PCR has gained acceptance due to its improved speed, sensitivity, reproducibility, robustness and the reduced risk of carry-over contamination. A quantitative, multiplex, real-time, fluorogenic PCR (TaqMan®) assay was developed in 2005 using probe-primer sets specific to *Ca.* Liberibacter spp., and a plant cytochrome oxidase (COX)-based primer–probe set as a positive internal control (Li et al., 2006). This assay allows for a real-time field diagnosis in less than 1 hour using a portable SmartCycler® (Cepheid, Sunnyvale, CA) machine and DNA extracts. The real-time PCR assay differentiates HLB strains by using different forward primers for each HLB strain but the same reverse primer and probe.

II. Equipment, Materials and Reagents

Equipment

1. Biological Safety Cabinet, Class II, Type A/B3 (new designation: Class II, A2)
2. PCR WorkStation
3. Smart Cycler® II – Cepheid
4. Cepheid Smart Cycler bench-top centrifuge (Cepheid # 900-0020)
5. Bench-top microcentrifuge capable of 14,000rpm
6. Vortex-Genie
7. Eppendorf Thermomixer (Brinkmann Instruments, Inc.)
8. Analytical Balance
9. Dedicated, annually-calibrated pipettors (P10, P50, P200)
10. Freezer (-20ºC, non-frost-free)

Materials

1. 1.7ml micro centrifuge tubes, clear and amber (pre-sterilized, certified DNase & RNase free, any vendor)
2. SmartCycler® tubes (25µl) (Cepheid #900-0022)
3. Sterile filter (barrier) pipette tips for the corresponding pipettes
4. Gloves
5. Disposable bench tissue paper (any vendor)
6. Absorbent disposable bench under pads (any vendor)
7. Microcentrifuge tube openers (any vendor)

C. Reagents

1. Molecular Grade (MG) water (any vendor)
2. TE buffer (any vendor)
3. Ethyl alcohol (200 proof)
4. Platinum® Taq DNA Polymerase (Invitrogen part # 10966-034)
   Note: Platinum *Taq* is supplied in a set with 10X PCR Buffer and 50mM $MgCl_2$
5. 10 mM dNTP mix PCR Grade (Invitrogen part #18427-013)
6. Primers and probes* (see tables for details) *Please note that probes take 7 to 14 business days to arrive once ordered.

All snap closure microtubes are to be opened using a decontaminated tube opener. Tube openers are decontaminated by soaking 2 hours in 10% bleach solution, followed by two rinses in water and diH_2O to remove bleach residue.

III. Real-time PCR Assay

A. Primers and Probes

Table 2. TaqMan primers

Primer Name	Primer Mix Name	Sequence 5' - 3' Synthesized by Integrated DNA Technologies, Inc.; Primer Purification = Standard Desalting	Target gene	Specific to
HLBas primer	**HLBas** primer Mix	TCG AGC GCG TAT GCA ATA CG	16S rRNA	Las
HLBr primer[1]		GCG TTA TCC CGT AGA AAA AGG TAG		
HLBam primer	**HLBam** primer mix	GAG CGA GTA CGC AAG TAC TAG	16S rRNA	Lam
HLBr primer[1]		GCG TTA TCC CGT AGA AAA AGG TAG		
COXf primer	**COX**[2] primer Mix	GTA TGC CAC GTC GCA TTC CAG A	COX	COX
COXr primer		GCC AAA ACT GCT AAG GGC ATT C		

[1] The HLBas and HLBam primer mixes share one common primer, HLBr.
[2] COX refers to a cytochrome C oxidase gene in plants and is based on the COX from Citrus
NOTE: The "r" in the primer name HLBr and COXr denotes the reverse primer.

Table 3. Probes for Real-time PCR (Synthesized by Integrated DNA Technologies, Inc.)

HLBp probe	56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ-1
COXp probe	5-TET/ATC CAG ATG CTT ACG CTG G/3BHQ-2

Note: probes take 7 to 14 business days to arrive, once ordered.

1. Tubes with lyophilized primers and probes (shown in Table 2 and 3) are centrifuged briefly (10-20 seconds at minimum 10,000 rpm) before opening to ensure that the lyophilized material is in the bottom of the tube.

2. Freezer stock solutions (100 µM) of the probes and primers
   Working inside a clean PCR workstation, primers and probes are re-hydrated to 100µM stock solution in 1xTE buffer and stored at - 20°C. Store freezer stocks of probes in amber-colored microcentrifuge tubes.

3. Working solutions of primer mixes
   Prepare a working solution of each of the **HLBas/HLBr, HLBam/HLBr** and **COX/COXr** primer mixes by adding 20 µl of the 100µM stock solution of the appropriate two primers of each pair mix to 960 µl 1xTE buffer (final concentration is 2 µM).

4. Working solutions of probes
   Prepare 1µM solutions of each probe in molecular biology grade water or 1x TE buffer by diluting the 100 µM probe stock solution (add 10 µl of stock solution to 990 µl of 1xTE buffer, final concentration is1 µM). Store small aliquots (i.e.: 20 µl) of probes at -20ºC in 1.5 ml amber-colored microfuge tubes to protect the primer from degradation due to light exposure. Small aliquots, suitable for one-day use, of probe are desirable since probes are sensitive to frequent freeze/thaw cycles.

B. Preparing the Real-time PCR Master Mix

Master Mix preparation and aliquoting must be done in a clean PCR workstation.

1. Label two sets of Cepheid 25 µl tubes for samples and positive and negative controls.

2. Prepare two Real-time PCR master mixes (Table 4) for the number of the samples plus additional (1 for every 10) samples, plus the 2 controls. Keep master mix on ice once prepared and prior to aliquoting for individual reactions.

Table 4. Real-time PCR Master Mix

Reagents	1 reaction	10 reactions	Final concentration
Molec. Grade water	4.7 µl	47 µl	N/A
10x PCR buffer	2.5 µl	25 µl	1x
$MgCl_2$ (50mM)	3.0 µl	30 µl	6.0 mM
dNTPs (10 mM each) (Invitrogen)	0.6 µl	5.0 µl	0.24 mM
Platinum Taq (5U/µl) Invitrogen	0.2 µl	2.0 µl	1 Unit
HLB primer Mix (2µM each)	3.0 µl	30 µl	240 nM
HLB probe (1µM)	3.0 µl	30 µl	120 nM
COX primer Mix (2µM each)	3.0 µl	30 µl	240 nM
COX probe (1µM)	3.0 µl	30 µl	120 nM
Total	23.0 µl	230 µl	N/A

NOTE: "HLB Primer Mix" in table refers to either HLBas/HLBr or HLBam/HLBr. COX primer mix refers to COX/COXr.

3. Pipette up and down to thoroughly mix, then aliquot 23.0 µl of Master Mix into each 25 µl Cepheid tube.

C. Sample or Control addition to real-time master mix

1. Take tubes containing master mix to the Cepheid cycler station and place all items on a new disposable lab mat.

2. Add 2 µl of undiluted sample (environmental) DNA to the corresponding Cepheid tube. The total reaction volume is 25 µl.

3. Each diagnostic run of the real-time PCR must include an HLB- positive (either Las or Lam as appropriate) DNA control and one negative control (negative template control, NTC). For the positive control reaction, add 2 µl of positive control DNA. Add 2 µl of MG water or TE buffer to the master mix for the negative control reaction.

   Note: Do not use 25 µl of master mix as your negative control. The negative control can indicate contamination of master mix reagents or water, or contamination introduced by the analyst during sample addition.

4. Close the caps and spin the Cepheid tubes in the Cepheid centrifuge for 10 seconds. Make sure no bubbles remain on the sides of the flat diamond-shaped area of the Cepheid tube. Place the tubes in the SmartCycler® in the appropriate I-core modules.

D. Loading Smart Cycler® and starting the run

1. Turn on the SmartCycler® Block first, followed by the SmartCycler® software. If not done in this order the machine will give an error message.

2. Program Set-up:
   - Stage 1: Hold at 95ºC for 20 seconds with optics off.
   - Stage 2: repeat 40 times and 2-Temperature Cycle.
     -the first temperature cycle, set 95ºC for 1 second with optics off
     -the second temperature cycle, set 58ºC for 40 seconds with optics on.

   Use a temperature ramping rate of 3 degrees per second.

3. To begin amplification: (instructions contained in the SmartCycler® manual)
   i. Select “Create Run”
   ii. Give the run a name (i.e.: date and protocol)
   iii. Select dye set (for this qPCR it is “FTTC25”)

iv. Select "add/Remove Sites" to select the protocol
v. Select the sites with the samples and click on ► symbol to add sites to the right column, and click on OKAY.
vi. Select "Start Run" found in the lower left-hand corner of the screen.

E. Assessment of the HLB qPCR using the SmartCycler® software Version 2

Analyze data from the run as per the thermocycler Manufacturer's instructions

- If the HLB-positive control sample DNA tests negative (FAM Ct = 0.00 or, or Ct 37-40), or your negative control tests positive (FAM Ct >0.00), the entire run is invalid and all samples must be retested.

- If the positive control DNA has a COX internal control TxR Ct = 0.00, the entire run is invalid and all samples must be retested.

- If an environmental (plant) sample has a COX internal control TxR Ct = 0.00 or Ct (37-40, the specific sample must be retested. If a retest again produces the same result, the sample must be re-extracted as that DNA does not pass quality control.

- Las or Lam positive samples: If a plant sample produces a FAM Ct value in the range of 0.00< FAM CT ≤36.0 the sample is determined to be positive for *Ca*. Las or Lam. (Note: The COX internal control for the real-time PCR must be acceptable. See Section E above.) Samples which test positive for HLB should be retested by real-time PCR to confirm the first run results. If a similar result is obtained, then the sample is determined to be positive. If this is a Potentially Actionable Suspect Sample (PASS), the sample must be forwarded for federal confirmation (see section F below).

- Negative samples: If a plant sample produces a FAM Ct =0.00 or ≥37 to 40 then it is determined to be negative for Las or Lam.

G. Samples Requiring Federal Confirmation

A Potentially Actionable Suspect Sample (PASS), in its simplest form, is a sample of a pest or pathogen of regulatory concern (including bacteria, viruses, nematodes, weeds, insects, mites, mollusks, etc) that have been presumptively identified by a laboratory without federal confirmatory authority.

The presumptive identification of a PASS by a non-approved laboratory would result in the sample(s)/specimen(s) or its biological extract (DNA, RNA, proteins, etc.) being forwarded to a designated Federal laboratory for confirmatory testing. Subsequent 'suspect' positive samples from within an APHIS-defined regulated area of the first PASS would not require federal confirmatory testing, but new finds outside of the defined area are considered a new PASS.

Typically, a sample considered a PASS would also encompass any sample that involves unusual or unexpected circumstances, for example, a new host, new location, an atypical biology, or potential bio-terrorism act.

'Suspect' HLB-positive samples from within a regulated area(s). A 'suspect' HLB-positive sample from within a designated regulated area is not considered a PASS. That means this category of sample does not require Federal confirmatory testing *unless* it represents a new host or a yet unconfirmed Liberibacter species in which case it must be forwarded to the PPQ laboratories in Beltsville, MD. Positive identification of *Ca.* Liberibacter asiaticus or L. americanus from samples that are not PASS would be conducted at those laboratories certified by CPHST to perform PPQ validated diagnostic assays for HLB.

'Suspect' samples from outside a regulated areas. A 'suspect' HLB-positive sample from any citrus plant or citrus psyllid(s) from outside the regulated area is categorized as PASS. That means this category of sample must receive full Federal confirmatory testing. 'Suspect' sample(s) that qualify as a PASS that are determine in laboratories certified by CPHST to perform PPQ validated diagnostic assays for HLB will require Federal confirmatory testing that includes identification on the basis of testing of DNA isolated from citrus plant tissue or citrus psyllids. Confirmatory testing of all PASS for HLB is conducted at the USDA PPQ laboratories in Beltsville, MD.

Any sample that is submitted (overnight delivery) to NPGBL must be accompanied by a PPQ Form 391 (Sample Submission Form), and Ct values for each run (see example table below).

Please provide a table of your real-time PCR data with the sample(s):

Example of real-time PCR Ct table to send to NPGBL

Sample ID	PCR Run #	Dilution	HLB FAM Ct Value	COX TET Ct Value
HLB-*state code*	1	Und	26.5	17.0

The NPGBL is authorized to receive a PASS sample for testing under to APHIS permit # 90015 for domestic suspect select agent plant pathogens.

Please provide NPGBL with the tracking number and estimated delivery date by e-mail (laurene.levy@aphis.usda.gov, renee.m.devries@aphis.usda.gov), prior to sending material to NPGBL at the shipping address below:

Dr. Laurene Levy
USDA-APHIS-PPQ-CPHST-NPGBL
BARC-East, Bldg 580
Powder Mill Rd
Beltsville, MD 20705,
Phone 301-504-7100 and fax 301-504-8539

If the submitting diagnostic laboratory held back a portion of a PASS and the PASS is determined to be positive for HLB, a select agent (SA), there is an follow-up action required of the submitting laboratory under the Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331). The submitting diagnostic laboratory is required to notify the APHIS Select Agent Program immediately and complete a SA form regarding the destruction or transfer to a SA registered entity. If you choose to destroy the remaining sample, it must take place within seven (7) days of results notification and a PPQ Office must witness the destruction of the sample on or before the 7-day period expires. See http://www.aphis.usda.gov/programs/ag_selectagent/index.html, or call 301.734.5960.

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8. SmartCycler® II Operator Manual Copyright 1999-2002 by Cepheid.

Document Revision History

Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	3-26-2007	To baseline the Diagnostic work instruction

Approved: Signature on File with Original Document

Date: 3-26-2007

Approved By: Renee M. DeVries, NPGBL Quality Manager
Approved By: Laurene Levy, NPGBL Technical Manager

This work instruction provides information on DNA extraction from psyllid samples collected from the field for the purpose of Citrus Greening or HLB (Huanglongbing) molecular detection by PCR. The extraction uses a commercial kit containing a spin column.

Related Work Instruction

WI-B-T-D-1 *Real-time PCR for Diagnostic Detection of Citrus Greening or HLB (Huanglongbing) from Psyllid Samples*

I. Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Homoptera: Psyllidae) is a pest of citrus and close relatives of citrus. It is found in tropical and subtropical Asia, Afghanistan, Saudi Arabia, Reunion, Mauritius, parts of South and Central America, Mexico, and the Caribbean. In the United States, Asian citrus psyllid was first found in Palm Beach County, Florida, in June 1998 in backyard plantings of *Murraya paniculata* (orange jasmine). By 2001, it had spread to 31 counties in Florida with much of the spread due to movement of infested nursery plants. In the spring of 2001, Asian citrus psyllid was accidentally introduced into the Rio Grande Valley of Texas on potted nursery stock (orange jasmine) from Florida.

Asian citrus psyllid damages plants directly through its feeding activities. While direct damage is serious, there is much greater concern that the psyllid is an efficient vector of the bacterium that causes the economically devastating disease called citrus greening or "Huanglongbing". Until recently, citrus greening was not found in the United States. In September 2005, infected citrus trees were found in Florida.

II. Equipment, Materials and Reagents

A. Equipment

1. Biological Safety Cabinet, Class II, Type A/3B (now referred to as II, A2)
2. FastPrep FP-120 or -24 instrument (Qbiogene, Inc) or Mini-bead Beater (BioSpec)
3. Balance, capable of weighing 20-100 mg
4. Thermomixer (Eppendorf #5350), or waterbath, capable of 56°C
5. Microcentrifuge, bench-top, capable of ≥10,000 rpm

6. Vortex

7. Freezer, -20, (non-frost free)

8. Dedicated, annually-calibrated pipettors (P10, P50, P200)

B. Materials

1. Qiagen® DNeasy Blood and Tissue kit (Qiagen #69504 for 50 tests or #69506 for 250 tests)

2. Sterile filter (barrier) pipette tips (P10, P50, P200)

3. Gloves (any vendor)

4. Weigh boats (any vendor)

5. Razor blades, single-use (any vendor)

6. Microcentrifuge tubes, 1.7 ml (pre-sterilized, certified DNase & RNase free, any vendor)

7. Paper towels, absorbent (any vendor)

8. Disposable, absorbent bench underpads (any vendor)

9. Ice

10. Microcentrifuge tube openers (any vendor)

11. Lysing Matrix A tubes (Qbiogene #6910-050)

C. Reagents

1. Ethyl alcohol (200 proof)

2. Phosphate Buffer Saline (PBS) buffer 1X, (pH 7.2), prepare (50mM potassium phosphate, 150 mM NaCl) or purchase commercially (i.e.: Technova, #P0200, 1000 ml)

Notes:

All non-screw tubes are to be opened using a decontaminated tube opener. Tube openers are decontaminated by soaking 2 hours in 10% bleach solution, followed by two rinses in water and diH_2O to remove bleach.

Packages containing psyllids must be opened inside a decontaminated Class II, Type A/B3 Biological Safety Cabinet, according to permit conditions.

Samples need to be stored securely at all times, and be accessible to appropriate personnel only.

If you have received packages from different states on the same day, process the samples of the first package completely (sections III and IV), then decontaminate the work area and change out all disposables prior to opening and processing the next package(s).

To minimize the potential for general contamination the DNA extraction area should be separated from the PCR work area.

To avoid cross contamination of sample, use a new razor blade, new gloves, new lab mats, and new weigh boats/paper with each sample.

III. Sample Processing/ Disruption of Psyllids

A. Preparation for DNA kit extraction

1. Prepare all Qiagen buffers according to manufacturer's instructions.
2. If not purchasing, prepare 1x PBS buffer.
3. If you are using the Qiagen Blood and Tissue kit for the first time, please read the Qiagen handbook, including the "Safety Information".
4. All centrifugation steps are carried out at room temperature (15-25 °C).

5. Vortexing should be performed as pulse-vortexing for 5-10 seconds.

6. Prior to using the kit's AL Buffer, ensure that there are no signs of precipitates in the bottle. If there are precipitates in the bottle, warm the bottle briefly by placing it into a beaker of warm (i.e.: 56°C) water for 5-10 minutes and then re-inspect the bottle to ensure that the precipitate has gone into solution prior to use.

7. Qiagen buffers AW1 and AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottle, to obtain a working solution.

8. Qiagen buffers AL and AWI contain guanidine hydrochloride which is not compatible with bleach. This is a hazardous chemical.

9. Buffer ATL is *not* required in this protocol, but is supplied with the kit.

10. Preheat the thermomixer to 56°C. Allow the thermomixer a few minutes to reach 65° C prior to placing the tubes into it.

B. Psyllid Drying

1. Place new absorbent paper under pad on a clean work surface.

2. Place each psyllid sample on its own new paper towel and allow to air dry for 10-15 minutes at room temperature. (Avoid drafty areas to prevent movement of pysllids.)

IV. DNA Extraction

1) Add 360 µl (a modified volume) PBS to each labeled Qbiogene Lysing Matrix A tube (2 ml).

2) Using a new 50-200 µl filtered pipet tip for each sample, wet the pipette tip with PBS, touch the wet tip to dry psyllids and transfer the psyllids to the tube. Each wet tip can carry up to 3 psyllids at a time.

For each psyllid sample received, place 1-5 adult psyllids into the Qbiogene tube. (Each 2.0 ml tube contains garnet matrix and one 1/4 inch ceramic sphere.) If the sample contains more than 5 adults, then prepare 2 Qbiogene tubes containing up to 5 adults each, labeled as subsample 'a' or 'b'.

3) Homogenize the sample using a FastPrep instrument (40 seconds at speed 6.0) or a Bio-Spec bead beater (time setting '2' at speed 48).

4) Centrifuge for 10 minutes at 14,000 rpm.

5) Pipet 180 µl of supernatant into a new, labeled, 1.7 ml microcentrifuge tube (not provided with kit).

6) Add 20 µl Proteinase K and 200 µl Buffer AL (*without added ethanol*).
   Note: Ensure that ethanol has not been directly added to Buffer AL.

7) Pulse-vortex 5-10 seconds, then incubate at 56°C for 10 minutes.

8) Add 200 µl Ethanol (96-100%) to each tube and pulse-vortex 5-10 seconds.

9) Pipet the mixture from step 9 (including any precipitate) into the DNeasy Mini spin column placed in a 2ml collection tube (provided in kit).

10) Centrifuge at ≥ 8,000 rpm for 1 min. Retain the spin column.
    Discard flow-though and collection tube.

11) Place the spin column in a new collection tube (provided in kit) and add 500 µl Buffer AW1.

12) Centrifuge for 1 min at ≥ 8,000 rpm. Retain the spin column.
    Discard flow-through and collection tube

13) Place the spin column in a new collection tube (provided in kit) and add 500 µl Buffer AW2.

14) Centrifuge for 1 min at 14,000 rpm. Retain the spin column.
Note: Handle spin column carefully to prevent column coming into contact with the flow-through.
Discard flow-through and collection tube.

15) Centrifuge for 2 min at 14,000 rpm.

16) Place the spin column in a new 1.7 ml microcentrifuge tube (you provide) and pipet 50 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute.

17) Centrifuge for 1 minute at $\geq$ 8,000 rpm to collect DNA elution.

18) Repeat elution (steps 15 and 16) again using the same collection tube containing the first elution flow through. You should collect a combined total of 100 µl DNA.

19) Store the DNA extract at 4 °C for immediate use only or at -20 °C to use in the future.

Document Revision History

Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	3-21-07	To baseline the work instruction
Revision	1	3-26-07	Changed title of this and the related work instruction (page 1). Reworded first paragraph on page 1. Clarified note on page 3. Changed title of section IV. Added BioSpec equipment information to step IV-4.

Approved: Signature on File with Original Document
Date: 3-26-2007

Approved By: Renee M. DeVries, NPGBL Quality Manager
Signature:

Approved By: Laurene Levy, NPGBL Technical Manager
Signature:

F

Citrus Greening Disease

Appendix F

Disinfecting Equipment

Introduction

Plant pathogens can persist on pruning shears, knives, and other implements used for cutting and pruning operations. Making a cut on an infected tree is sufficient to contaminate the cutting tool; subsequent cuts on other trees will introduce the viroid and infect the tree. Viroids (small pieces of RNA similar to a plant virus but lacking the protein coat) are extremely difficult to remove from tools and are not inactivated by most disinfectants or high heat.

Instructions

Use the following instructions to disinfect equipment.

A 5% solution of liquid household bleach (sodium hypochlorite) will inactivate citrus viroids and prevent their spread. To use, spray the cutting surface of tools with the bleach solution; or, briefly immerse tools in it.

Use a fresh bottle, since bleach solutions deteriorate. Household bleach sold for use in laundering clothes is a 3-6% solution of sodium hypochlorite at the time of manufacture. If the concentration is greater than 5%, then dilute with water.

Select a thick-walled, non-breakable plastic container, with a top opening large enough to easily dip the cutting surfaces of the pruning shears, and fill with sufficient bleach solution to cover tools. Alternatively, fill a spray bottle with bleach solution and use it to spray tools. Replace the bleach solution every 2—3 hours, since it deteriorates quickly when exposed to air.

When sampling is completed for the day, disinfect the shears by dipping in the bleach or spraying, then rinse thoroughly with water. To minimize the corrosive effects of the bleach on the pruning tool, dry it after the water rinse and coat the cutting surfaces with a thin film of lubricating oil.

CAUTION: Household liquid bleach is very corrosive. Avoid contact with eyes, skin, or clothing. Follow all precautions on the label.

Appendix G

Insecticides

TABLE G-1 Insecticides registered for control of psyllids on citrus

Trade Name[1] and Percent AI[2]	Active Ingredients	EPA Reg. No.	Usage
Marathon® II (21.4%)	Imidacloprid [1-[(6-Chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine]	3125-549-59807	Ornamentals, fruit and nut trees, and vegetable plants in greenhouses, nurseries, and interior plantscapes
Marathon® 60 WP (60%)	Imidacloprid	3125-492-59807	Ornamentals and vegetable plants in greenhouses, nurseries, and interior plantscapes
Tame 2.4 EC (30.9%)	Fenpropathrin	59639-77	Commercial use on indoor and outdoor ornamental and nursery plants
Dursban® 4E (44.8%)	Chlorpyrifos [0,0-diethyl 0-(3,5,6-trichloro-2-pyridyl) phosphorothioate]	655-499	Fruit, nut and citrus trees, golf course turf and commerical nursery plants
Discus™ (2.94%)	Cyfluthrin [(RS)-a-cyano-4-fluoro-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate)] (0.70%) and Imidacloprid	432-1392-59807	Ornamentals, non-bearing fruit and nut trees, in field and container nurseries
Chlorpyrifos G-Pro 4 (44.7%)	Chlorpyrifos	79676-9	Commercial nurseries and greenhouses; golf course turf, turf and ornamentals around industrial buildings; turf and ornamentals in road medians

1 Other products might be registered for control of citrus psyllids. Check with APHIS–Environmental Services for more information.

2 AI = Active ingredient