United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine

Guignardia citricarpa (Citrus Black Spot, CBS) technical working group final report

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United States Department of Agriculture (USDA)
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Center for Plant Health Science and Technology (CPHST)

Technical Working Group Report

Infections considered highly suspect for Citrus black spot (CBS), caused by *Guignardia citricarpa*, were found on March 8th, 2010, in Collier County, Florida. The infections were found on Valencia oranges by an inspector of the Florida Department of Agriculture (FDACS), Division of Plant Industry (DPI), responding to a request from a grower to survey four grove blocks in the Immokalee Grove (TRS 47-29-34). DPI's Gainesville laboratory detected the presence of citrus black spot, *Guignardia citricarpa*, on March 29 and two University of Florida (UF) Laboratories confirmed the identification.

After conducting polymerase chain reaction testing of the submitted tissue and DNA samples from Florida as well as sequence analysis, APHIS' National Plant Germplasm and Biotechnology Laboratory and Molecular Diagnostics Laboratory in Beltsville, Md., confirmed the presence of *G. citricarpa* in the samples, corroborating with FDACS-DPI's and UF plant pathology laboratory's initial diagnostic results. United States Department of Agriculture Animal Plant Health Inspection Service Plant Protection and Quarantine Citrus Health Response Program (USDA APHIS PPQ CHRP) in cooperation with Florida Department of Agriculture and Consumer Services Division of Plant Industries (FDOACS DPI) has surveyed all groves within 7 miles of the infested Immokalee groves, all the groves along the highways between Immokalee and the orange juice processors, all lemon groves in the state and all groves that have associations with the infested groves. A total of 11 grove blocks were determined to be positive within a 4-mile radius in Collier County (near Immokalee) and one new grove multiblock in Hendry County, 14 miles northeast of the initial finds. (The TWG considers this to be a significant development, making surveillance during the summer more important.) More than 95% of the fruit has been harvested from citrus groves in Florida, which is a big limitation for making many conclusions out of this years' survey. State and Federal CHRP personnel are stationed at all the juice-processing facilities and all positive detections have been traced back to known infected groves. State DPI personnel are checking citrus along routes used by trucks loaded with citrus from the known infested area, and for delivery to processing and fresh market packinghouses. As a result of these detections and the subsequent technical questions that arose, the State Plant Health Director and the Director of Emergency Programs at PPQ asked that the Center for Plant Health Science and Technology (Part of USDA APHIS PPQ) to develop a technical working group (TWG) to answer technical questions that arise.

On April 23rd 2010, the initial technical working group meeting was held via teleconference with participants from Argentina, Australia, South Africa and the United States. During this teleconference, the members were introduced and the charge to the TWG was provided. Several questions were asked and answers are presented in Appendix 1. Members of the TWG are presented in Appendix 2 and a list of references is provided in Appendix 3. The following are recommendations were compiled from this initial teleconference in answer to the technical questions asked of the TWG:

Recommendations of the Technical Working Group:

- **Establish quarantine areas.** Quarantine areas should include all infested groves and surrounding groves that fall completely or partially within a minimum of 1 mile of an infected tree. This may be a conservative distance for a quarantine, but justifiable given how little information about the infested area is currently known. Once more information is known about the nature and size of the infestation, smaller quarantine areas may be warranted.
- **Work with University of Florida Institute of Food and Agricultural Sciences (IFAS) to determine specific fungicide regimes, detection methods and appropriate best management practices (esp. Leaf litter cleanup) during summer months**.
    - **Require litter cleanup in the quarantine areas.** Clean up should require either burning the material or covering the leaf litter evenly with several cm of soil. Do not limit litter cleanup to those groves known to be infested, as incipient and latent infections in groves that did not test positive cannot be detected readily since most fruit has been harvested. Leaf litter clean up should be required in all groves in the quarantine areas and continued on a weekly basis. To acquire additional information on pathogen distribution during the summer months prior to fruit ripening, sample the leaf litter before destruction, allow symptom development under controlled conditions and extract DNA from developing lesions to determine if *G. citricarpa* is present.
    - **Check any citrus fruit on ground during cleanup for the presence of black spot.** If symptoms are present these fruit should be tested using validated molecular diagnostics for the presence of *G. citricarpa*.
    - **Utilize spore trapping technology to detect *G. citricarpa***. Several different models are available. Use validated molecular diagnostics to differentiate *G. citricarpa* from G. *mangiferae*.
    - **Move USDA mobile laboratory to Immokalee or work closely with laboratories at the IFAS Research stations in Immokalee and Lake Alfred, FL**. This will provide support for high throughput of leaf litter, abandoned grove and fallen fruit samples for identification of *G. citricarpa*.
- **Begin surveying lemon fruit 20-28 weeks after petal drop**. Latent fruit infections may be induced to form fungal structures through the use of warm incubation, treatment with the growth regulator ethephon and high light intensity.
- **Survey abandoned groves in Florida for the presences of CBS.** Abandoned groves should still have fruit and may be more easily surveyed.
- **Ensure that all groves within Florida are surveyed prior to harvest this year**. This should be completed during the later stages of fruit development, optimally within 30 days of harvest. By doing so, full delimitation of the infested area can be determined.
- **Eradication may be possible but eradication feasibility will only be known after full delimitation.** If and only if the infested area is small and contained can eradication be accomplished. Focus early efforts on determining the full distribution of the disease. This will require an additional year of survey to fully delimit the truly infested areas (see above).
    - Eradication (if possible) would include the destruction of all infested host material (uprooting and burning) in the quarantine area and a buffer around the infested areas;
    - A host free period of 2 years (Citrus appears to be the only host of *G. citricarpa*, unlike *G. mangiferae*);
    - Replanting with certified disease free nursery stock.
- **Ensure that all nursery stock utilized for replanting is as free as possible from black spot and other citrus diseases**.

Appendix 1. Notes from the 23 April 2010 Black Spot of Citrus Technical Working Group meeting.

Situation Report by M. Hornyak: United States Department of Agriculture Animal Plant Health Inspection Service Plant Protection and Quarantine Citrus Health Response Program (USDA APHIS PPQ CHRP) in cooperation with Florida Department of Agriculture and Consumer Services Division of Plant Industries (FDOACS DPI) has surveyed all groves within 7 miles of the infested Immokalee groves, all the groves along the highway between Immokalee and the orange juice processor, all lemon groves in the state and all groves that have associations with the infested groves. These investigations have revealed no additional CBS positives. CHRP personnel are stationed at all the juice-processing facilities and have not detected positive fruit from any other groves.

Since the initial TWG meeting teleconference, a new grove 14 miles away from the initial find was determined to be positive. The grove is located on a small county road that is a commonly used route to six different orange juice processors. The positive tree was located adjacent to the road. The grove has been completely surveyed and this appears to be the only positive tree in the grove.

Several of the participants would like to see an international workshop on Citrus black spot occur at the 2011 Joint APS/IPPC meeting in Hawaii.

1) What can be done during the summer to detect CBS?

Little has traditionally been done during the summer months after fruit has been harvested as fruit symptoms are by far the easiest method of disease detection.

South Africa monitors the inoculum load in orchards that are used commercially with the Kotze-Quest inoculum monitor. Inoculum data can provide information on the ascospore load in the area but is unable to distinguish between *G. mangiferae* and *G. citricarpa* ascopores without molecular tests. However, these data provide valuable information to more effectively apply fungicide. Spore counts may not always be a good tool to establish infection periods since rain events can wash spores out of the air, thus negatively impacting spore counts, but may be the perfect time for infection to occur.

Fruit symptoms develop late in the season making detection difficult. Also, if fruit is not handled properly cold storage and processing, symptoms may develop postharvest. Incipient fruit infections can develop more fully postharvest, especially once the fruit are removed from cold storage.

Abandoned groves can be surveyed. These groves are most likely to still contain fruit and will not have been sprayed with fungicides for control of CBS or other diseases, although there are none within 10 miles of the current infested area. Fallen fruit may be present in areas where harvests have already occurred and should be considered during the summer for surveys.

Symptoms of latent infections have been induced in fruit as early as 20 and 28 weeks after petal fall in Brazil, by warm incubation and treatment with the growth regulator ethephon (Baldassari, 2007). Lemons can be surveyed earlier in the fall than other citrus and in Brazil, lemons show foliar symptoms more commonly than sweet orange. Leaf symptoms in lemon (and more so in sweet orange) are indicative of a very severe black spot problem.

In Brazil, ascospores formed in perithecia from decomposing citrus leaves are the primary inoculum sources for the disease cycle and is important for tree to tree spread. The ascospore is a sexual spores spread by wind and is responsible of medium to long distance dispersal between trees. Movement between trees of up to 25 m was observed in orchards a minimum of 14 years old. Llarger trees in these orchards were a physical barrier, so the ascospores didn't go longer distances. In São Paulo State irrigation is uncommon and tree canopies are close the ground. In orchards less than 4 years old, trees have more air circulation and ascospores could move longer distances. The conidia of *Guignarida citricarpa* are asexual spores and spread chiefly by water. The conidia are an important part of within-tree (secondary) disease spread.

There is much information available through the multi-pest surveys conducted by the Citrus Health Response Program. *Guignardia citricarpa* and citrus black spot is one of the diseases that surveyors were specifically trained to identify in April 2009. Many groves were surveyed this year after the training while ripe fruit was present. Additional information (map-based) is is provided in Appendix 4.

In São Paulo State conditions, the first symptom that is found in the orchard at the initial onset is hard spot. After the disease is present in the majority of trees in the block, false melanose begins to show. Hard spot symptoms commence when the fruit starts to break color. False melanose symptoms are expressed 4 months after petal fall. Fruit is susceptible to infection during the first 5 months and always susceptible to symptom development, but the incubation period for symptom expression is variable. When the fruit is small, symptom expression takes longer, with symptom expression being shorter for larger fruit.

Australia did a study where they collected leaf litter and examined the occurrence of leaves with perithecia and pycnidia. They found that a site with a high incidence of infected fruit (90% the previous season) corresponded to leaf litter with 1-3% of leaves with perithecia in late spring (October to December in Australia), with levels peaking to 15% mid-late summer (January and February, 120 leaves were sampled and examined per week). In low disease incidence blocks (<10% fruit infection) the spike in % leaves with perithecia was not observed. In the low-disease incidence blocks, collecting leaf litter was more effective than using spore traps for population monitoring, but was a tedious process requiring a large minimum number of leaves (approximately 10,000 for the whole study).

Kiely (1948) outlines a technique for the development of symptoms from latent infection in green citrus leaves. Unfortunately, *Guignardia mangiferae*, also present in Florida, is difficult to differentiate from *G. citricarpa* morphologically (both ascospores and conidia) and are best distinguished molecularly. This will complicate any monitoring process that is utilized. A real-time PCR assay has been successfully used on symptomatic leaf litter in Australia and similar techniques have already been used in the US on fruit symptoms and may be transferable to other plant tissues. These methods are currently being evaluated by CPHST National Plant Germplasm and Biotechnology Laboratory (NPGBL)

Research conducted in South Africa has provided methods to monitor nurseries and effectively sample symptomless trees using PCR. Researchers have established protocols on surveying orchards and verifying symptoms on branches, as well as monitoring the soil for the presence of the pathogen, collecting leaf litter under host trees and monitoring spore populations in orchards. A number of protocols have been developed that are currently being utilized to establish and maintain official pest-free areas for movement of fruit to the European Union. Other PCR protocols also have been developed for the detection of *G. citricarpa* isolates from other regions (e.g. Brazil and Argentina) (Peres, 2007) (Bonants, 2003). Thus, several forensic identification protocols based on PCR are available. Scientists at CPHST are in the process of collecting these protocols for evaluation and use in the field as appropriate.

A feasibility study is underway to see if a forensic analysis of *G. citricarpa* can be undertaken at the NPGBL. The analysis will utilize the isolates from Florida and will also obtain isolates from Australia, South Africa, Argentina and Brazil for comparison. Scientists at CPHST were on the call to establish collaborative efforts with their international counterparts. Australian scientists have done basic DNA sequencing and have some isolates that will be provided to CPHST. South African scientists have also done some DNA sequencing of the *Guignardia citricarpa* and have a good collection of isolates. Pedro Crous (formerly of the University of Stellenbosch now at the CBS Fungal Biodiversity Centre in Utrecht, The Netherlands) is also a good contact and is interested in *G. citricarpa* and currently working on assessing *Guignardia* biodiversity. George Carroll (Oregon) is working on sequencing and classification of *Guignardia* and would also be a good resource.

2) How far away from a positive find should surveying occur?

Epidemics caused by *G. citricarpa* do not tend to spread rapidly, typically only a few meters a year under natural conditions. It has been hypothesized that *G. citricarpa* ascospores would be dispersed within a 6m radius, similar to that of *Venturia inaequalis* (Sposito, 2007). The main method of long distance pathogen dispersion is via movement of infected foliage on trees being moved long distances such as nursery stock or on leaf debris in loads of harvested fruit.

Focus should be put on citrus nurseries and containing leaf litter during fruit transport as the most likely pathway for disease movement. Also, fruit destined for export out of the region should be stored at cold storage temperatures and treated with a fungicide regime in the field to prevent the potential spread of the disease by fruit and leaf litter movement (Agostini, 2006). Although fruit are not considered a pathway for disseminating the disease, this mechanism should not be overlooked especially with new geographical introductions. For this reason, QoI fungicides registered for postharvest fruit applications may also help limit the movement of infected fruit because the fungicides are locally systemic and may inhibit the development of quiescent infections.

In Brazil, it took 12 years for the pathogen to naturally move from the initial detection site in Rio de Janeiro to the main citrus producing area in São Paulo.

The most likely pathway for disease spread in São Paulo State was through the movement of infected nursery plants when the system had open nurseries. It is important to trace back symptomatic trees to the nurseries from which they were purchased when possible and determine if other trees sold by that nursery during the same time period are diseased. Nursery plants are very difficult to survey because the leaves are asymptomatic and samples needed for PCR are so small (~180mg of tissue). Therefore, nursery surveys would need to be random and consist of a very large number of samples to effectively detect the pathogen. The disease is most likely to have come to São Paulo State by two means: 1) from Rio de Janeiro by little-leaf tangerine with leaves attached to the peduncle of fruit because in Brazil this tangerine is sold as such; and 2) from illegal propagative material from other countries. As far as we can ascertain (as well as in other parts of the world), the fruit is not the primary inoculum source for disease cycles.

3) What control methods should be utilized?

- In Florida, the recommendations from the University of Florida have been to reduce leaf litter by using irrigation to increase decomposition in addition to applying 5-6 foliar applications of one of the 2 approved fungicides (copper or strobilurins/QoI fungicides).
    - South Africa found that leaf litter management by leaf removal and mulching allowed a reduction of up to 97% CBS development. It is necessary to get a huge reduction in spores because the pathogen produces spores in large quantities.
- In Australia, the use of an integrated approach using litter removal, fungicide and good canopy management (skirting, selective limb removal, removal of prunings from the canopy and orchard) has been shown to reduce infected fruit to ≤1.7%.
- Using drip irrigation in nurseries would drastically reduce development of the disease in nurseries because you no longer have favorable environmental conditions. Other conditions that might lead to a reduction in wet foliage warrant consideration.
- A systemic fungicide with activity to prevent quiescent infections from developing in harvested fruit needs to be determined. A long-term residual fungicide such as an EBDC fungicide should be registered to protect fruit during the growing season. Postharvest fungicides recently registered in the US on fruit (QoI and phenylpyrrole) may be used to reduce possible movement of the disease via transportation of fruit. Additional fungicides will be needed and registration of new products for post-harvest and pre-harvest should be secured. There is also the potential for a local needs

exemption (24(c)) that might be secured for nursery use of Topsin. This would greatly reduce the potential for leaf infection and therefore spread potential.

4) If the infested area turns out to be small and relatively contained, should eradication be attempted? If so, how would you suggest this be done?*For your answers, assume that only the science is an important part of the decision process.*

Eradicate by removal of trees in the orchard and continue to monitor and use protective treatments.

----------------If a high level of confidence can be reached that the infested area is small and contained, the most likely approach to achieving successful eradication is the complete destruction of all host material in the quarantine area (uprooting and burning), followed by a host-free period, and then re-establishment with disease-free nursery stock. As the pathogen can infect twigs, removal of only fruit and leaves may be insufficient for eradication. It would be reasonable to hypothesize that citrus are the only hosts of *G. citricarpa*, and the records outside of citrus are probably *G. mangiferae* but misidentified as *G. citricarpa* (in Australia two herbarium records from the 1970's of "*G. citricarpa*" were found on banana and avocado, but these are likely *G. mangiferae*). No one is aware of any molecularly-confirmed examples of *G. citricarpa* on a host outside of *Citrus*. The duration of the host-free period should be sufficient for all leaf litter to decompose (minimum of 2 years?). The host-free period should include ample time to ensure citrus surrounding the quarantine area, if any, can be surveyed to support the absence of the disease prior to replanting the quarantine area; preventing re-establishment of the disease within the quarantine area. Sufficient time should also be left to ensure re-establishment is not adversely affected by root rot pathogens able to survive on any decomposing root material remaining from the tree destruction – in keeping with good horticultural practices. In the case of citrus canker in Queensland, the host free period was a minimum of 2 years.

Eradication of black spot will not be easy. Both ascospores and conidia can infect fruit, leaves and branches, but the symptoms are only readily observed in fruit. Therefore, it is difficult to discern which trees are diseased until the symptoms appear in fruits. The asymptomatic but infected leaves and branches can be inoculum sources, when the leaves drop and begin to decompose and when the branches die. Disease symptoms may not be present until many years after the initial infection in a grove.

A big challenge is confidently delimiting the spread of the pathogen; due mostly to the latency of the pathogen and climate.

Some considerations:

<u>Pros</u>

1. The growers in the infested area were willing to discuss and cooperate with the eradication option if scientifically sound.
2. CBS spread is slow.
3. Suppression while the decision's practicality is determined is possible.

4. Much more citrus in the US, Caribbean and North America is now at greater risk, so there is much to gain (assuming we have reliable geographic range data from this part of the world).
5. No serious harm or yield reductions on processed fruit when control regimes are in place. Fungicides used judiciously can manage this disease quite nicely. Even organic growers have some management options.
6. The disease is easy to survey for when symptoms are present.
7. Fresh fruit from infested areas presently face some marketing restrictions (but the biological basis for those regulations is not sound because pycnidiospres are not normally primary inoculum).

Cons

1. Eradication of CBS has never been done or even attempted. It may not work.
2. We have only approximate data on how far ascospores can really travel and remain infective.
3. Aside from Ghana, the FL climate is probably among the most black spot conducive of any commercial citrus region in the world (?)
4. Additional management costs are one more blow to the economic stability of this industry.
5. Other diseases (HLB in particular) pose a potential defeater for any successful black spot eradication.
6. The 4-6 month "blind" period during the season makes survey and monitoring more difficult.
7. We don't know how it got here initially, so reintroduction prevention is haphazard.

An effort should be made to very actively suppress the disease (*i.e.* destroy all fallen leaves as soon as possible and continuously during the summer in the known infested area as much as possible) until the time of symptom onset in the fall of 2010. This should be coupled with an effort to monitor ascospore release in the known infested area and in several other areas of the industry during the summer of 2010. During this period, we should gain a better understanding of the true geographic range of the disease in Florida. Then, if the geographic range appears to be still quite contained, in the Fall or early Winter of 2010, the decision could be made about removing trees in the known infested areas and within about 25 meters of any tree that once showed or is now showing symptoms. If after one season, considerable progress toward elimination of CBS can be documented and no major tropical storm events blow leaves far and wide from the infested area, another round of suppression and tree removal could be warranted.

If the identified infected zone is contained and small, we should quarantine the area and destroy the inocula. We should initiate a nursery certification scheme.

The area we are considering is already more than 15 sq. mi. Since *G. citricarpa* moves surreptitiously in leaf litter and on nursery stock, it has probably already spread to other areas and eradication is not likely to be feasible. Thorough surveys are not possible at this time since a lot of the fruit has already been harvested. It is also good that there are no nurseries in that area. Certainly everything possible should be done to suppress the disease, but eradication would involve too much destruction, be very costly and politically unfeasible. The biology of this pathogen and limited spread under its own power would seem to lend itself to an eradication program.

If an eradication program is started, monitoring of both adjacent areas and sentinel plants planted farther away should be done over the next couple of years to ensure that there have been no escapes to other growing areas. Monitoring should include automated sampling systems such as spore samplers, regular surveys (especially in adjacent growing areas) for fruit symptoms by trained staff on susceptible hosts, and molecular testing or culturing of all suspect samples.

Appendix 2. Citrus Black Spot (CBS) Technical Working Group Members

Jim Adaskaveg, University of CA, Riverside
Juan Pedro Agostini, INTA, Argentina
Russ Bulluck, USDA APHIS PPQ CPHST
George Carroll, University of Oregon
Megan Dewdney, University of FL
Osama El-Lissy, USDA APHIS PPQ EDP
Tim Gottwald, USDA ARS
Megan Henderson, USDA APHIS PPQ CPHST
Charla Hollingsworth, USDA APHIS PPQ CPHST
Paul Hornby, USDA APHIS PPQ
Michael Hornyak, USDA APHIS PPQ
Lise Korsten, Univ. of Pretoria, South Africa
Laurene Levy, USDA APHIS PPQ CPHST
Andrew Miles, Tree Path. Centre, Australia
Mary Palm, USDA APHIS PPQ MDL
Natalia Peres, University of FL
Tim Schubert, FL DOACS DPI
Don Seaver, USDA APHIS PPQ CPHST
Marcel Bellato Spósito, Fundecitrus, Brazil
Pete Timmer, UF, Professor Emeritus
Kurt Zeller, USDA APHIS PPQ CPHST

Appendix 3. References

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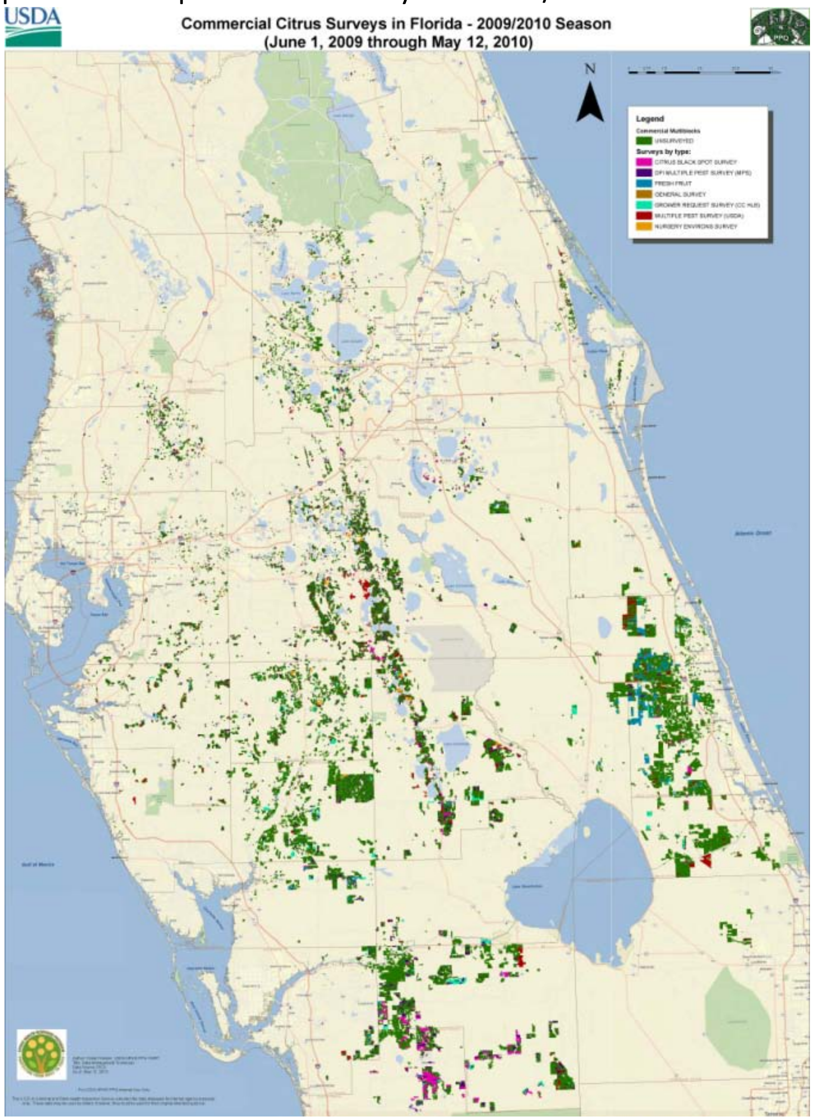
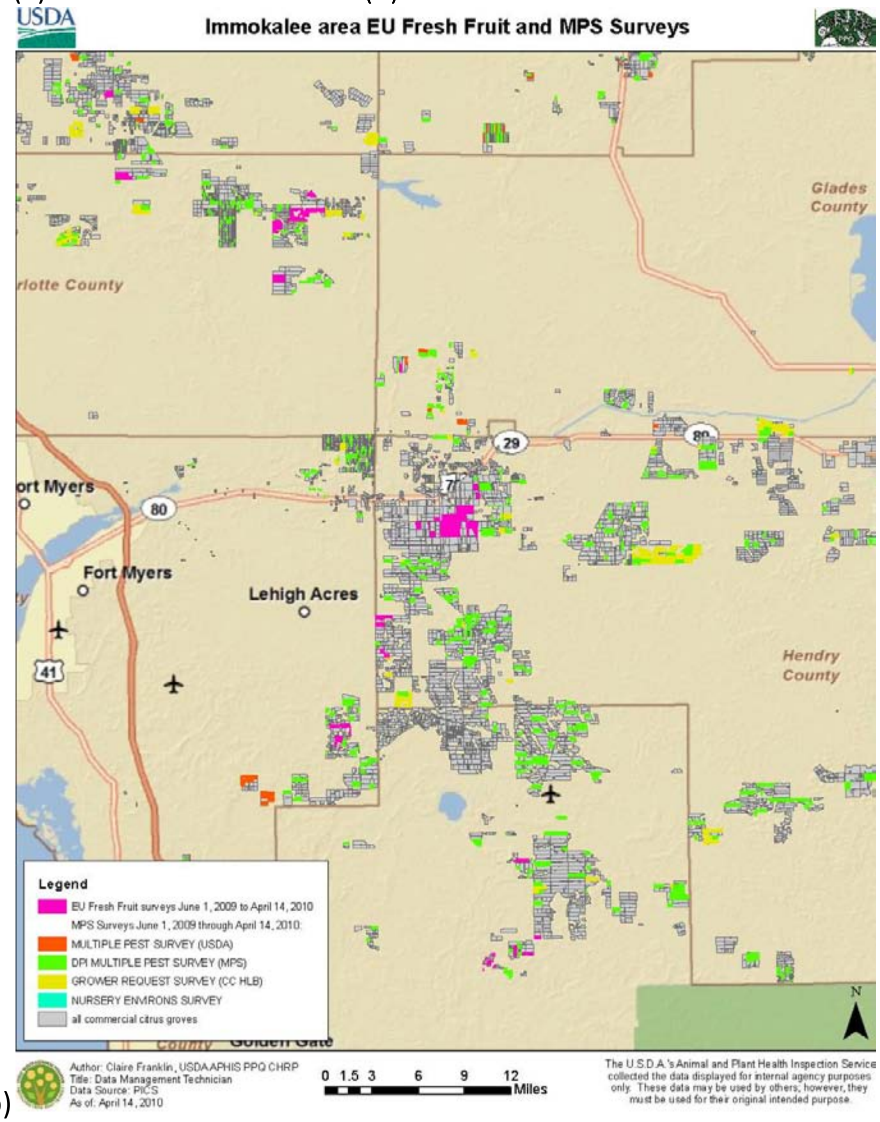
Appendix 4. Maps of citrus surveys for 2009/2010 season in Florida (a) and around Immokalee (b).



(a)



(b)