United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine
CPHST Plant Epidemiology and Risk Analysis Laboratory

Survey Protocol in Exclusionary Facilities for the Interstate Movement of Citrus and Other Rutaceous Plants for Planting from Areas Quarantined for Citrus Greening, Asian Citrus Psyllid, and Citrus Canker

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Background:
APHIS has developed this survey protocol in support of the APHIS Citrus Nursery Stock Interim Rule (Docket# APHIS-2010-0048) for exclusionary facilities (USDA, 2010a) to detect the presence of Citrus Greening (CG), Asian citrus psyllid ACP, and Citrus Canker (Xcc) in Citrus Nursery Stock (CNS). CNS for interstate movement has inspection, sampling, and testing requirements (USDA, 2010b). Visual inspections must be performed by APHIS certified specialist every 30 days for ACP, CG, and Xcc, (USDA-FDACS, 2006-2007; USDA, 2008b). Inspection of citrus tissues is the best way to survey for ACP (Halbert, 1998) and Xcc (Schubert, 2001).

Detection Survey for ACP: Visually inspect new flush growth of CNS. Use an aspirator to collect psyllids and place them in alcohol. Submit suspect specimens to a USDA approved identifier. Inspection can be augmented by using yellow or blue sticky cards or suction traps which are inspected every 30 days. Place yellow or blue sticky cards at 0.5 meters in height on or within preferred host plants (USDA, 2008b). A minimum of one sticky card per 1,000 ft^2 of growing space is required (http://ohioline.osu.edu/hyg-fact/1000/1033.html).

Detection Survey for Xcc: This bacterial disease causes distinct necrotic and raised lesions on the leaves, stems, and fruit (Schubert, 2001) and CNS must be inspected for Xcc when taking samples for CG (protocol below). Visual inspection is sufficient for Xcc because the disease causes distinct necrotic and raised lesions on the leaves, stems, and fruit (Schubert, 2001). Furthermore symptoms appear as early as seven days after infection which aids early detection. Leaves from plants showing symptoms of Xcc upon inspection must be tested by at an APHIS certified laboratory approved by APHIS using an APHIS protocol (http://www.aphis.usda.gov/plant_health/cphst/npplap.shtml).

Detection Survey for CG: Symptoms of CG alone are not diagnostic. Other plant pathogens or abiotic conditions (soil, nutrients, weather, etc.) can cause similar symptoms. Therefore, in addition to a 30-day visual inspection cycle, CG plant tissue sampling and testing must be performed by APHIS certified specialists and APHIS approved laboratories using an APHIS

protocol (http://www.aphis.usda.gov/plant_health/cphst/npplap.shtml). Sampling and testing is required every six months.

Recommendation for CNS shipped from a CG quarantine area: The period between transmission of the pathogen by psyllid vectors or plant propagation and the appearance of visual symptoms varies depending on the time of year of initial infection, environmental conditions, tree age, species/cultivar, plant health, etc. (Aubert, 1987; Catling, 1970; Gottwald *et al.*, 1989; Gottwald *et al.*, 2007). The incubation period before symptom expression varies from two months to five years (Gottwald *et al.*, 1989). The first occurrence of visual symptoms can be dramatic in some trees, but subtle in others. As a precaution, we recommend that lots of plants intended to be shipped interstate must be subjected to at least two sampling and testing cycles, including mother plants, with negative results. The first sampling and testing occurs six months after propagation and the second sampling and testing occurs one year after propagation. This is necessary because achieving a successful PCR test requires time for the pathogen to increase in the plant sufficiently so that bacterial cells can be detected molecularly (Gottwald *et al.*, 2007; Hung *et al.*, 2001). A defined lot of CNS systematically sampled[1], tested, and found negative for CG at six and twelve months after propagation meets the testing requirement for partial or whole shipment for an additional six months after which another sampling and testing is required to maintain shipment eligibility during the following six months. The Technical Working Group (USDA, 2010c) discussed a sampling and testing method (unpublished) with a 1% detection level (detects a disease prevalence of $\leq$1%) of CG in CNS with no greater than a 5% probability of not detecting a pathogen in a population of plants (confidence interval). Sampling and testing requirements change as the detection level and confidence interval fluctuate. Sampling continues every six months until the entire inventory has been depleted.

An example of the sample sizes and the number of laboratory tests required for different size lots of CNS for interstate shipment are given in Table 1. Each CNS lot could be produced in a single greenhouse with or without compartments, and applies to facilities with 1 to 3,500 or more plants. The maximum sample size is 300 plants because when the population is greater than 3,500 no detection efficiency can be gained by increasing the sample size.

Sampling Method

The sampling method demonstrated in Table 1 assumes that the validated laboratory testing protocols (http://www.aphis.usda.gov/plant_health/cphst/npplap.shtml) are 95%% accurate

[1] Systematic sampling is useful to sample large numbers of plants in a defined lot when the sample number is known (Table 1). Samples are chosen at a prescribed interval and the start of the sampling interval is chosen at random. For example, the sample number for a defined lot of 1,000 plants is 265 (Table 1). The sampling interval is determined by (1,000 divided by 265 multiplied by a random number between 0.01 and 1. In Microsoft Excel® the formula for this example is: [=1,000/26]*[=RAND()]. The sampling sequence could be: Plant #3, #7, #11, #15 ---- 996#. The sampling sequence will change each time a new random number is inserted into the formula. Sampling may also be conducted at random numbering each plant and choosing the plants at random and no plant is sampled more than once.

(Hocquellet *et al.*, 1999; Li *et al.*, 2006). If testing is less than 95% accurate then the sampling method can be easily adjusted. The SAS JMP 8.0.1® spread sheet that generated Table 1 can accommodate any detection level and testing accuracy desired (available upon request). The 1% prevalence is used to explain the method.

Examples to explain Table 1

1. A grower has a 1,001 CNS plants for shipment interstate. Under compliance agreement (USDA, 2010b) a grower wishing to ship sub-lots of plants for the foreseeable future must first complete the two sampling and testing cycles (minimum of one year), including mother plants, with negative results. The first and second cycle would each require that 275 plants be tested in the lot. If 200 plants are then sold, after six months another systematic sampling and testing of 265 plants of the remaining 801 is necessary. If 400 plants are then sold, a systematic sampling and testing of 230 plants of the remaining 401 is necessary for the next six months of shipment. Testing occurs every six months until the inventory is exhausted.

2. A grower has a defined lot of more than 3,500 plants for shipment interstate. Under the compliance agreement (USDA, 2010b) the grower wishing to ship sub-lots of plants for the foreseeable future must first complete the two sampling and testing cycles (minimum of one year), including mother plants, with negative results. The first and second cycle would each require that 300 plants be tested in the lot. Every six months the sample size remains at 300 until number of plants is reduced to less than or equal to 3,500. After that the sample size is found in Table 1. Testing occurs every six months until the inventory is exhausted.

Table 1. Sample size[1] and laboratory test requirement lots of CNS for interstate shipment that consists of one to greater than 3,500 plants.

Lot Size[2]	Number of Plants to Sample[2]	Number of Laboratory Tests Required[4]
1 to 100	All plants	1 to 25
101 to 200	101 to 160[3]	26 to 40
201 to 300	195	49
301 to 400	220	55
401 to 500	230	58
501 to 1,000	265	67
1,001 to 1,500	275	69
1,501 to 2,000	280	70
2,001 to 2,500	285	72
2,501 to 3,000	290	73
3,001 to 3,500	295	74
> 3,500	300	74

[1] Sampling and testing are designed to detect a prevalence of CG that is $\geq$ 1/100 (USDA, 2008a). The probability of not detecting a population of plants with a 1% prevalence of CG is no greater than a 1/20 (5%).

[2] Sample size (column two) is adjusted for convenience and does not adversely affect the detection level desired.
[3] To meet the 1% detection level all plants in a lot of up to 160 plants must be tested.
[4] Laboratory tests are run on mature leaves (Hung et al., 2001) taken from one to four CNS using a protocol approved by APHIS (http://www.aphis.usda.gov/plant_health/cphst/npplap.shtml). Thus four or 166 samples would require one or 41 laboratory tests.

Literature

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