Protocol for Collecting and Handling Plant and Soil Samples for the Detection of Plant Parasitic Nematodes at the Nematology Laboratory, Plant Pest Diagnostic Branch, California Department of Food and Agriculture

A Detailed Overview

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Exterior Quarantine Samples: Plants sampled from shipments entering California from other US states and countries. Currently, the California Department of Food and Agriculture (CDFA) has a single external quarantine program, namely, the Burrowing and Reniform Nematode Exterior Quarantine that targets the mentioned nematodes especially from US States with known infestations of those nematodes. While the program is aimed specifically towards the detection of the Burrowing Nematode and the Reniform Nematode, all plant parasitic nematodes present in a sample are identified by State Nematologists.

The California Plant Quarantine Manual section 3271, appendices A–D provides certification requirements including sampling for the quarantine nematode species. Appendix D provides guidelines for prioritizing sampling of commodities based on their intended use. The majority, if not all plant and soil shipments received through the Burrowing and Reniform Nematode Exterior Quarantine are indoor decorative, a lower priority than plants intended for farm and non-farm outdoor planting. However, especially in Southern California, indoor decorative plants are routinely planted outdoors, and thereby, gain a higher priority.

All incoming shipments are to be held for inspection by the respective county. The nursery stock is issued a "Quarantine Warning Hold Notice" at a California agricultural inspection station to the destination county where it is held for inspection until released by the County Agricultural Commissioner.

The following sampling protocol is followed for incoming plant shipments:

Preparation

1. Equipment. Hand trowel, hand shears, disposable latex or rubber gloves, plastic bags, a bucket, a cooler, cold packs preferable wrapped in paper, a spray bottle containing 70-90% ethanol (disinfectant), water-resistant marker pen, plant damage and pest detection report forms (PDR form 65-020), county forms, a clipboard, paper towels (for clean-up), a bag for disposing garbage.

2. Obtain and review list of plants shipped.

3. Visually inspect entire lot of plants prior to sampling. All plants are to be loaded off the trailer/truck and made available for inspection

4. Determine sample size based on lot size. A minimum of 5 plants or 5% of the first 200 plants, and an additional 2% of lot size over 200 plants.

Example:

Size of lot	Collect soil and roots from *at least*:		
1-5	All plants	=	1 composite sample
6-109 (5%)	5	=	1 composite sample
110-129 (5%)	6	=	1 composite sample
130-149 (5%)	7	=	1 composite sample
150-169 (5%)	8	=	1 composite sample
170-189 (5%)	9	=	1 composite sample
190-200 (5%)	10	=	1 composite sample
300 (5% + 2%)	12	=	1 composite sample
400 (5% + 2%)	14	=	1 composite sample

Collection

5. Composite samples within a plant species by variety, and/or container size. Collect soil and roots in bucket, mix thoroughly, extract at least 1quart sample for laboratory analysis.

    a. Select plants at random, however, if compositing varieties and container sizes, ascertain that a sample includes roots and soil from all varieties and container sizes within a lot. Include soil and roots from any plant that may appear less thrifty in growth than the remaining plants and display off-color, stunting, or other above ground symptoms indicative of an impaired root system. Do not limit sample to only plants that look "unhealthy".

    b. <u>Collect soil and root samples from 4-6 inch and 10-14 inch containers</u> by carefully inverting and unpotting plants. Plants in large containers may be packed in organic or lava rock potting mixtures. Unpot the plant and collect roots and adhering soil mainly from the inner mass of root growth. Avoid collecting only peripheral roots on soil surface and inner perimeter of the container.

6. <u>Sampling seedlings in plugs and flatbed containers:</u> destructive sampling of entire seedling plants is required. Collect at least 1quart soil and roots. Do not separate top growth from plant.

7. Sampling bare roots and rootless cuttings: Sample should consist of any root primordia or root nubbins, any aerial roots, or the basal section cut from the cane to include at least one node.

8. Sampling postal shipments of plants: A minimum 1quart sample is ideal but not always possible when dealing with a postal, or other shipment of few seedlings (~<10) with sparse root growth. In such a case, it is suggested that at least 15% of the plant shipment is sampled. At the very least, collect as much of a sample as possible.

Export Quarantine Samples:
Plant shipments for export often require freedom from certain plant parasitic nematode pests in order to fulfill the phytosanitary quarantine (PQ) requirements of various countries. Most often these shipments comprise plant seeds, and PQs call for freedom from nematodes pests that either dwell internally within seeds, on the seed surface or as contaminates in seed debris. For example: The Stem and Bulb Nematode, *Ditylenchus dipsaci* within seeds of grain and vegetable crops, The White tip of Rice Nematode, *Aphelenchoides besseyi* on hulls of paddy rice, The cyst nematode, *Heterodera schactii* as cysts in grain and vegetable seed debris. Occasionally whole plant seedlings and bulbs are also exported and may require freedom from the same nematode pests.

Sample size

Sample size for seed is based on shipment size. Contact the CDFA Nematology Laboratory for assistance. Agricultural county official is responsible for collecting samples after inspecting the respective shipment.

1. Grain and vegetable crops: the Nematology Laboratory requests a seed sample of 1-5% of the shipment size when the shipment is less than 500 grams.

2. Grain crops such as, alfalfa, onion, garlic (except paddy rice): when seed shipment size is more than 500 grams, usually several kilograms, minimum sample size is 250 grams.

3. Vegetable crops: when shipment size of seeds is more than 500 grams, usually several kilograms, minimum sample size is 150 grams.

4. Paddy rice: according to the USDA-CDFA protocol developed for exporting paddy rice to Turkey, the minimum sample size for laboratory analysis is 10 grams sub sample collected from a larger composite sample of at least I kg, however, larger sample sizes (up to 250 grams) are encouraged.

Collection

1. Grain and vegetable seeds: seed samples must be collected randomly from the top, middle and bottom levels of a container, sack, bin, or storage facility. Collect from at least five random spots at each level when sampling large seed shipments stored in large facility or container. Collect at least 1 kg from each level, mix thoroughly, and from this mixture collect the required amount (see sample size above) to send to the Nematology Laboratory.

2. Paddy rice seeds: the protocol for sampling paddy rice for the detection of the White-Tip of Rice Nematode is given below.

    i. Determine the size of the shipment in metric tons (MT) [1 MT = 2204.61 lbs; 1 oz = 28.34 g; 1 cwt = 100 lbs.]

    ii. If size of shipment = 1,000 MT then,
    Collect 5 samples each = 1 kg from top location (= 5 kg total)
    Collect 5 samples each = 1 kg from middle location (= 5 kg total)
    Collect 5 samples each = 1 kg from bottom location (= 5 kg total)

    Total number of samples collected = 15
    Total weight of samples collected = 3x 5 = 15 kg

    iii. Thoroughly mix each 5 kg samples separately (i.e., mix top amount separate from middle, separate from bottom amount).

    iv. From each well-mixed 5 kg sample collect a 1 kg sample

    Total number of reduced samples = 3
    Total weight of reduced samples = 3 x 1 = 3 kg

    v. From each separate well-mixed 1 kg reduced sample collect a sub sample of 10 g minimum weights. Send this sub sample to the Nematology Laboratory for analysis. Amounts above the minimum required weight is encouraged (50–80 g).

    Total number of sub samples sent the Laboratory = 3
    Total weight of final samples sent to the Laboratory = 3 x 10 = 30 g (minimum)

    vi. For each additional 1,000 MT of shipment paddy rice
    Collect 1 kg sample. Additional samples are to be collected first from the top location, then the middle, and then the bottom locations. This means: for the first additional 1,000 MT collect 1 kg from the top; for the

second additional 1,000 MT collect from top and middle, and so on. No more than 1 kg is pulled from any of these locations.

vii. Thoroughly mix each location's (top, middle and bottom) samples separately. Collect a 1 kg reduced sample from each well-mixed sample and, subsequently, collect a 10 g minimum weights sub sample for laboratory analysis.

Nursery Samples:
The California Code of Regulations (CCR) section 3640 (b) mandates that nursery stock for farm planting shall be commercially clean with respect to economically important nematode species detrimental to agriculture. "Commercially clean" is defined as "pests are under effective control, or present only to a light degree, and that only a few plants in any lot or block of nursery stock, or on the premises, show any infestation or infection." In accordance with this regulation, and for the purpose of commodity certification, CDFA's Nursery Inspection Procedures Manual (NIPM) states clear guidelines for sampling nursery plants under different situations.

Nematode sampling protocol for nursery stock in containers, field, and bare-root

1. a) Field grown nursery stock – generally
Collect root and soil samples on a 40 x 40 ft grid. Composite samples on an acre or nursery stock variety basis. This will result in 28 sub samples.

b) Field grown nursery – special case
At the discretion of the Department, root and soil samples may be collected on an 80x 80 ft grid interval and composited on a two acre basis when:

i) The planting site has been treated at the product label rate for the kind of nursery stock being produced.

ii) No nematodes have been found in the previous 2 successive nursery crops on the site.

c) Delimitation
Collect samples on a 20 x 20 ft grid interval and composite on a quarter acre basis.

2. Container, flat, and frame grown nursery stock

Collect a composite sample of soil and roots from one plant in every 100 square feet of bench or frame space.

Delimitation sampling is not done in this case.

3. Sampling bare roots

Sample varieties separately. Determine lot and sample size from the "Sampling table for examination of bare-root nursery stock for nematodes", given below.

Number of plants, bundles or boxes in lot	Sample size
2-8	2
9-15	3
16-25	5
26-40	7
41-65	10
66-110	15
111-180	25
181-300	35
301-500	50
501-800	75
801-1,300	110
1,301-3,200	150
3,201-8,000	225
8,001-22,000	300
22,001-110,000	450
110,001-550,000	750
>550,000	1,500

Directions for use:

Step 1. Find the total number of plants in the lot in the left column. The corresponding sample size in the right column gives the number of plants to sample.

Step 2. Find the total number of bundles, boxes or containers in the lot, in the left column. The corresponding sample size in the right column gives the number of units from which to collect samples.

Step 3. Divide the number obtained in Step 1 by the number obtained in Step 2. The result is the number of plants to sample in each of the units in Step 2.

Commercial and Residential Samples:
Two other nematode detection programs of lesser magnitude than the quarantine and nursery programs include the detection of economically important plant parasitic nematodes associated with plants grown and established in California soil either commercially, or in residential/dooryard situations. Commercial situations may include soil and plant samples collected from fields, orchards, vineyards and row crops. Residential situations may involve samples taken from individual plants, home lawns, vegetable gardens, potted plants and pre-plant soil beds to diagnose plant growth problems at private residences.

Depth of sampling

Most plant parasitic nematode species are concentrated in the upper 30 cm from the soil surface**.** However, the horizontal and vertical distribution of plant parasitic nematodes may vary largely with plant type, root growth, climate, agronomic conditions, cultural practices, and nematode species. In general, soil and root samples of annual crops should be taken to a depth of 12-18 inches. Deep-rooted perennial crops can be sampled to 36

inches. Also, samples collected after a prolonged fallow should be taken to a depth of 36 inches. Certain nematode species, such as, the Root-knot Nematode, the Pin Nematode (*Paratylenchus hamatus*), the Dagger Nematode (*Xiphinema index*), the Needle Nematode (*Longidorus* spp.) infest deep rooted hosts and hence, require deep sampling up to 18-36 inches.

Collection

1. *Trees*
   Collect soil samples from the canopy edge, and include feeder roots.

2. *Vineyards and row crops*
   Collect soil and roots from within rows, where root growth is undisturbed by tillage and cultural practices.

3. *Commercial field*
   a) Obtain background information: location, soil type, notable symptoms, cropping history, last nematicide treatment, and other growth limiting factors.

   b) Stratify field: subdivide field according to observable variations in previous growth, soil texture, moisture and drainage patterns, and cropping history.

   c) Randomly collect soil and root samples representing each stratum or entire field. Random collections may be taken in a zigzag pattern to ascertain coverage in fallow fields, established row/field crop, or vineyards and orchards.

Sample size

1. *Field*
Depending on area and crop being sample, collect a composite soil and root sample comprising of at least 20 cores or sub samples for 5 or less acres of uniform field or soil conditions. Mix sub samples thoroughly in clean bucket. Extract at least 1quart sample size for laboratory analysis.

2. Home garden/individual plants
Collect composite sample of at least 1 quart of soil and feeder roots for laboratory analysis. Mix sub samples thoroughly.

Time of sampling

Samples should be taken when soil is moist, less than 60 centibars, when nematodes are expected to be active.

Care and Handling of Samples:

1. Put raw, non-processed sample in durable polyethylene (plastic) bag only. Use two bags for durability, if necessary. *Above ground plant parts* **(**foliage, stems) decompose rapidly after collection and, therefore, should be kept in bags separate from *soil and root samples. Seed samples* also should be put in plastic bags. Paper bags for seed samples tend to tear over time and handling during transit to the processing laboratory.

2. Label plastic bag containing samples. Attach all identifying tags and papers to the outside of the bag. Moisture from the sample will destroy any labels and documents enclosed within the bag.

3. Do not rough-handle samples. Certain nematodes (the Stubby root, Dagger, and Needle Nematodes) are very susceptible to abrasions caused by roughly handled samples. These nematodes may lose their mobility, or even be killed if a bagged soil sample is thrown or dropped from a height. Handle samples carefully by treating them as perishable commodities.

4. Samples should be kept cool and moist after collection. Direct sunlight and excessive heat (as in the trunk of a car) should be avoided**.** Brief exposure to temperatures of 35-40 C or above, is sufficient to kill several nematode species. *Store sample at 10-15 C (50 F).* Storage of tropical soils at low temperature (5 C) may affect the recovery of some nematodes. Once collected, samples should be processed as soon as possible for the best recovery of active nematodes. Ideally, a sample should be processed within two days of collection, however, when it is not possible; they should be stored at cool temperatures. Vegetative samples tend to deteriorate rapidly if held long in storage, even at cool temperatures. On the other hand, storing soil and root samples a few days may allow for egg hatch and an increase in nematode numbers thereby, increasing the chance for nematode detection. *Use and insulated cooler to store samples during on-site collection. If a frozen refrigerant package ("Blue Ice") is used to keep samples cool during transportation to the processing laboratory, then wrap the package in a few folds of paper to prevent chill injury of sample through direct contact.*

5. Do not wrap roots in moistened paper towel. Roots wrapped in moistened paper may hinder the effective extraction of nematodes and may promote fungal growth and sample decay.

6. Care in packaging small amounts of root sample. When only a few roots are present in a nursery sample add some potting mixture/soil to the roots in a plastic bag to prevent dehydration of the sample during storage and transportation to the processing laboratory. When only a few roots are present in a exterior quarantine sample without soil, and no processing laboratory exists at the County office, the roots should be put in a nematode sample vial with one or two drops of water and sent by overnight mail to the Nematology Laboratory. More than one vial per

sample may be used as long as proper and complete designation is given. When the root sample is large, it is best to put roots with one or two drops of water in a plastic bag. Avoid large air spaces by sealing (tying) the bag close to the enclosed sample.

7. Do not send seed samples that have been chemically treated. Chemically treated seed samples will be rejected by the laboratory and a request for a sample of non-treated seeds will be issued.

8. Make certain that all information is given in the PDR submission.

9. If possible, send rush samples by overnight mail.

10. Mailing samples. When shipping same type of sample to more than one laboratory, *keep Nematology sample and PDR form separate from the other laboratories' samples and forms. Do not mix samples*. The Nematology Laboratory does not share samples with other laboratories and nematode extraction procedures use the entire sample submitted.

To submit samples for nematode diagnoses, or for questions, please contact: *Nematology Laboratory, Plant Pest Diagnostics Branch, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448, or call the Nematology Laboratory at 916-262-1100, fax number: 916-262-1190.*

Submission of Interstate Samples for Plant Parasitic Nematode Diagnoses at the Plant Pest Diagnostics Branch, California Department of Food and Agriculture

Summary

1. *Sampling*: the sampling schemes detailed in the overview may be used appropriately in other states that plan to send non-processed soil and plant samples to the Nematology Laboratory for nematode diagnoses. The sampling schemes provide guidelines for sampling containerized plants, seedlings, seeds, field crops and soil, bare roots trees, and row crops in nursery, commercial and residential situations. *A minimum sample size of 1quart soil and roots is required.*

2. *Bagging non-processed soil and plant samples:* samples must be placed in durable single or double plastic bag. See procedure for sending root samples in "Care and Handling of Samples" in overview.

3. *Processed samples:*
    a. *Heat-killed and preserved nematodes in 2.5% formaldehyde solution* may be sent for diagnoses to the laboratory. Heat kill nematodes in water suspension in a glass or hard clear plastic vial, by adding an equal volume of boiling water to a volume of live nematodes in water suspension, followed by enough formaldehyde to make up a 2.5% solution. For example: in a 22 ml vial containing about 10 ml of nematode suspended in clear water, add 10 ml of boiling water, shake, add about 5 drops of 37-40% formaldehyde, shake, and securely cap vial.

    b. *Mounted nematode specimens*. Permanently mounted nematodes in dehydrated glycerin, or temporary mounts in 2.5% formaldehyde must be packaged in slide mailers for the Nematology Laboratory. When few specimens (less than 10) of a suspect nematode species are present in a suspension, it is best to send the nematodes heat-killed and preserved in 2.5% formaldehyde as specimens in temporary slides tend to shift to the perimeter of the cover slip during transit to the Nematology Laboratory. Seal temporary slides with clear nail polish.

4. *Sample identification documents and labels*. All bagged samples, vials and slides must be fully labeled providing an identification number that corresponds to the accompanying documents. Information required per sample must include: state of origin, address, host, date of collection, situation (nursery plants, field, orchard, row crop, tree, etc.), symptoms, number of specimens (vial, slide), suspect nematode species (optional), name of contact person, and additional remarks. See "Care and Handling of Samples" in overview.

5. *Mailing shipment*: non-processed soil and plant samples must be sent to the Laboratory in cardboard boxes or insulated Styrofoam containers. A chilled refrigerant package wrapped in newspaper must accompany the samples.

Similarly, slides in mailers, and suspensions in vials may be boxed and sent by express or overnight mail to: *Nematology Laboratory, Plant Pest Diagnostics Branch, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448*. For questions contact the Nematology Laboratory at the above address, or phone number: 916-262-1110; fax number: 916-262-1190.

6. *Service Fee*: The Nematology Laboratory currently charges per sample, $ 20 for processing, and $20 for nematode diagnostics. A total of $40 per sample is charged for both processing and diagnostics.