Soil and Container Mix Protocol

Protocol for Detecting *Phytophthora ramorum* in Soil and Container mix
Revised November 2010

See http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/ for latest approved protocol.

Soil or container mix infested with *Phytophthora ramorum* appears to the unaided eye exactly the same as non-infested soil or container mix. Therefore, all soil and container mix samples must be handled carefully. All tools used to collect soil or container mix samples must be disinfested with a solution of 10% bleach, quaternary ammonium at the labeled rate, or full-strength Lysol™ spray, then rinsed thoroughly with distilled water and allowed to dry. Alternatively, tools may be flame-sterilized with a propane torch between sample collections. All soil and organic material should be removed from the tools prior to being disinfested. Care should be taken to not transfer soil or container mix from one sample location to another on shoes, gloves, or clothing. All sampling equipment should be cleaned and disinfested prior to entering a new nursery sampling location or block. Care must be taken to ensure that soil or container mix, within a sampling location, are not cross-contaminated in any manner. To reduce the risk of contamination, samples should be collected starting with the least infested area and moving toward the most infested area.

Definitions

Aliquot – a volume of substrate from a composite sample that is placed into a container and assayed; usually three aliquots (approx. 50-150 ml or 2-5 oz) from each composite sample are baited.

Block of plants – A contiguous group of host and associated plants with less than a 2 m (6.5 ft) break of non-host plants or empty space.

Composite sample – A mixture of subsamples that are physically combined to form a single representative sample from a designated area.

Container mix – Soilless substrates (also referred to as growing media) placed in pots and used to grow plants; usually consists of bark and peat but also may contain slow-release fertilizer, sand, vermiculite, perlite, etc.

Lot – Set of plants that can be identified or grouped by shipment, cultivar, or production unit.

Soil – Substrates in the field on which potted plants are located; often this consists of peat and bark fines washed from pots, plant debris, gravel, or any combination of these.

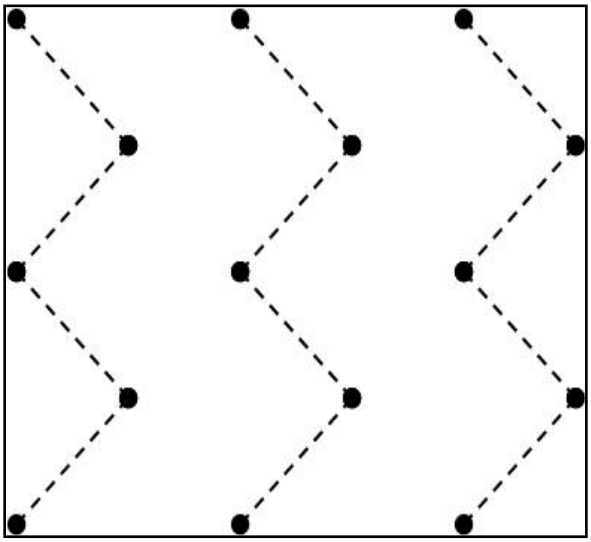
Subsamples – Small amounts of soil or container mix that are combined to form a single, composite sample; collecting subsamples increases the chances of finding *P. ramorum* if it is present.

Sampling Soils and Container Mixes

Sample Collection

1. Record the physical location (address) of the nursery site along with GPS reference coordinates. (***Tip****: If a GPS unit is not available, many cellular phones have GPS capabilities. Also Google Earth can be used to obtain coordinates using an address.)*
2. Prepare or secure from the facility manager/owner, a diagram of the nursery or sampling area, which includes row or block numbers and plant species/cultivars. If possible, collect reference GPS coordinates for each block of plants.
3. Each 1 L (1 qt) composite sample should consist of a ***minimum*** of 15 subsamples collected from soil or container mix within the targeted area. Subsamples should be collected in zigzag transects according to the pattern in the diagram below (Figure 1).

Figure 1. Recommended pattern for collecting subsamples of soil or container mix to produce a representative composite sample



4. Referencing Table 1, collect composite samples from both soil and container mix for each block of plants. An exception to this would be if all plants (including container mix and pots) were destroyed or the plants are not on a soil substrate (e.g., concrete or asphalt). Each sample should contain approximately 1 L/1 qt (volume) of soil or container mix and be placed in a 4 L (1 gallon) size zip-to-close plastic bag. The number of composite samples collected will depend on the size of the block of plants being sampled (Table 1).

Table 1. Number of composite samples to collect per block

Survey Area Size		Composite Samples of Soil	Composite Samples of Container Mix
(m^2)	(ac)		
<1,000	<0.25	5	5
1,000-2,000	0.25-0.5	10	10
2,000-4,000	0.5-1	20	20
>4,000	>1	30	30

Note: *If the soil surface is covered with gravel having a large amount of plant debris on top, collect as much debris as practical. If the gravel has little plant debris on top, collect subsamples from the soil beneath the gravel. If water permeable weed block (landscape cloth) is present, either covered with gravel or under gravel, small slits should be made in the cloth to allow for sample collection.*

Soil

a. Collect a representative composite sample (approximately 1 L (1 qt)) from the surveyed area (e.g., a block of plants, a nursery bed, a shade house, etc.) with a trowel to a depth of approximately 5-10 cm (2-4 in). *(**Tip**: If soil is loosely packed, a plastic spoon can be used to collect sample. The spoon can then be sealed in the corresponding sample's plastic bag for easy disposal in the laboratory. This method is NOT recommended for sampling container mix because substrate at the bottom of pots cannot be sampled.)*

b. Samples should be collected from around and under pots containing plants suspected of being infested or infected with *P. ramorum* or from areas where diseased plants were previously located. This may require scraping soil from on or under nursery cloth or anything else on which pots are or were located.

c. Place each composite sample into an individual plastic bag; if the soil is wet or saturated from rain or excessive irrigation, double bag the slurry to avoid leaks.

Container mix

d. Collect a representative composite sample from each block of plants using a wide bore soil tube (highly recommended); one core from each or every other pot in the block of plants is sufficient depending on the number of pots present.

5. For each composite sample, break up clods and root masses, then thoroughly mix the sample in the bag; this can be done in the field or laboratory.

6. Moisten the sample with distilled water if it appears dry, as desiccation will severely affect the ability to recover *P. ramorum* from a soil sample.

7. Disinfest sampling tools and soles of shoes (e.g., 10% bleach, quaternary ammonium at the labeled rate, or full-strength Lysol™ spray) between samples to prevent potential dissemination of the pathogen. Next, thoroughly rinse tools with distilled

water to remove all disinfestation product residues or allow tool to dry. *(**Tip**: Rinsing off disinfestation residues and allowing the tool to dry prevents possible sterilization of your next sample. Distilled water can be purchased at most grocery and big box stores.)*

Sample Transport, Storage, Shipping, and Processing

1. Place all samples in a cooled, insulated ice chest for transport to the laboratory or until samples are shipped. If samples cannot be shipped immediately, hold them in a refrigerator or cold room (4-10°C/39-50°F) for a maximum of two days.
2. Before shipping, samples should be double-bagged using gallon-size zip-to-close, self-sealing plastic bags, making sure to clearly label each bag using a permanent marker. To further protect samples, each sample can then be placed inside a 2 L (2 qt) disposable storage container which is also clearly labeled using a permanent marker. PPQ 391 forms (http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/PPQ_Form_391.pdf) should be filled out completely and placed inside a separate zip-to-close bag and placed in the same box as the samples.
3. Contact laboratory personnel before shipping to advise them that a sample will be arriving. Ship samples via overnight courier. Avoid shipping on Fridays and prior to holidays to avoid shipping delays that may compromise the quality of the sample.
4. Samples should be sent to a qualified state laboratory or to a USDA-APHIS-PPQ regional laboratory (see below).

Eastern Region: Grace O'Keefe
USDA-APHIS-PPQ
Pennsylvania State University
105 Buckhout Lab
University Park, PA 16802
Phone: 814-865-9896
Email: Grace.Okeefe@aphis.usda.gov

Western Region: Craig A. Webb, Ph.D.
USDA-APHIS-PPQ
Dept. of Plant Pathology
Kansas State University
4024 Throckmorton Plant Sciences
Manhattan, KS 66506-5502
Phone: 785-633-9117
Email: Craig.A.Webb@aphis.usda.gov

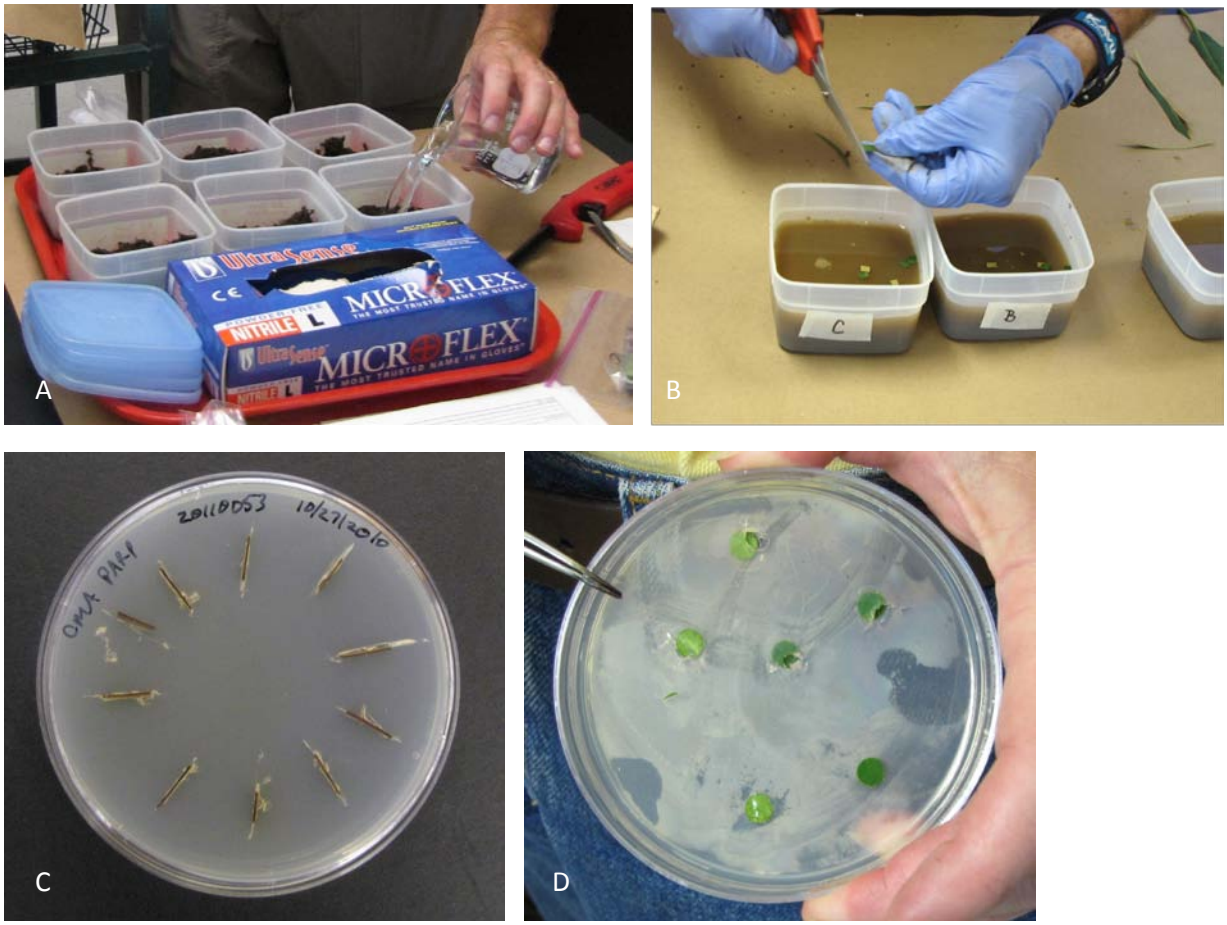
Procedure for Baiting Soil and Container Mix Samples *(S. N. Jeffers, Clemson University, 2010)*

1. Once samples arrive in the lab, they should be protected from exposure to direct sunlight near windows. If samples cannot be processed immediately, they should be stored in a refrigerator or cold room (4-10°C/39-50°F). Do not permit the samples to freeze or dry out.
2. If the soil or container mix sample is desiccated when it arrives in the lab, moisten with distilled water, re-seal the bag, and allow the moistened sample to sit for 48-72 hours before processing.

3. Thoroughly mix the 1 L (1 qt) sample within the bag, breaking up any clods. Divide the sample equally into two 500 ml (17 oz) aliquots, placing one sample into a new sealed zip-to-close bag or container then placing it in cold storage (4-10°C/39-50°F) for a minimum of 30 days.
4. Prepare and label three containers (e.g., small 0.5 L (1 pt) plastic containers, resealable 1 L (1 qt) bags, etc.) for each composite sample to be baited.
5. Thoroughly mix the remaining sample, then place an aliquot approx. 1 to 2 cm (0.5 to 1 in) deep into each of the three containers; soil deeper than this may inhibit zoospores from swimming to the surface.
6. Add distilled water to a depth of 2.5 cm (1 in) above the soil surface; stir the mixture and allow it to settle. ***Note****: organic debris may continue floating.*
7. Bait leaves should be free of blemishes, damage, disease, and pesticides. *Rhododendron catawbiense* and/or *Camellia japonica* are recommended bait types (if both bait types are available it is recommended to use both). Using a standard hole-punch or scissors, prepare enough leaf pieces (~10/container) to bait all containers (Figure 2). Leaf pieces cut with scissors should be approximately 5 mm (< 0.25 in) across. *(**Tip**: Use different-shaped leaf pieces to differentiate between bait types if two types of bait are being used.)*
8. Using sterile forceps, add 8-10 leaf pieces of each bait type (or 15-20 leaf pieces if only using one bait type) to each container. Baits should float on the water surface. If some of the baits sink, do not remove them, instead add additional baits. Cover containers to avoid evaporation and desiccation.
9. Store containers at 18-22°C/64-72°F for three days (an incubator maintained at 20°C/68°F or a closed cabinet works best).
10. For each container, remove six baits of each host type (or 12 baits of one host type) with sterile forceps and blot dry on a clean paper towel. Dispose of paper towels after each sample.
11. Place the six bait pieces of each host type from one container on a separate plate of PARPH-V8[1] medium (e.g., one plate with six rhododendron leaf pieces and one plate with six camellia leaf pieces, or alternatively, two plates of same host tissue baits) so they are embedded completely in the agar. Leaf pieces placed on the agar surface will dry-out and curl up. There should be six plates and 36 baits from each composite sample: 3 containers × 2 plates/container × 6 baits/plate (Figure 2). *(**Tip**: Baits can be inserted into the medium vertically, which prevents shadowing during microscopic examination. To prevent media tearing, a scalpel can be used to make small incisions where baits are going to be placed. Vertical placement may require slightly thicker agar or smaller bait pieces, however the plates no longer have to be read on both sides.)*

[1] See Appendix A for recipe

Figure 2. Baiting soil samples and plating baits *(images courtesy of Grace O'Keefe, PPQ and Jennifer Falacy, Washington State Dept. of Ag)*



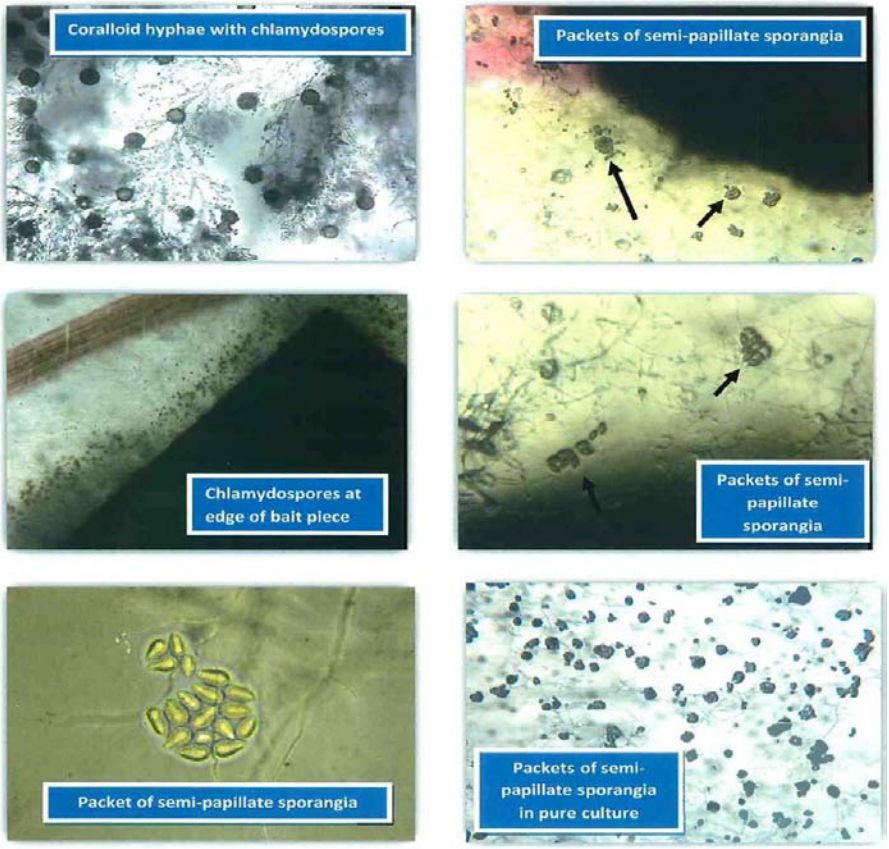
A) Preparing soil samples for baiting. B) Baiting soil samples using Rhododendron and Camellia leaves. C) Plate with baits inserted vertically. D) Plate with baits inserted horizontally.

12. Place plates upside-down in a plastic box or zip-to-close bag to prevent desiccation; incubate plates at 15-20°C/59-68°F in the dark for up to 28 days; a designated incubator works best but a closed cabinet in an air-conditioned room can also be used.
13. Using a dissecting or inverted microscope, examine plates frequently (starting 2 days after baits have been plated) for colonies that resemble *P. ramorum* – i.e., those with typical coralloid hyphae, large golden chlamydospores, and packets of semi-papillate sporangia on the surface (Figure 3); mark these with a permanent marker or grease pencil.
14. Once colonies form on plates, follow the morphological identification protocol found at http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/cultureprotocol6-07.pdf
15. *P. ramorum* hyphae often are visible 2-5 days after baits have been plated. However, patience, persistence, and good observational skills often are the keys to finding *P.*

ramorum on the isolation plates; *P. ramorum* may be recovered from only one of the 36 bait pieces and may not be recognizable until several weeks after baits are plated.

16. Subculture isolates to fresh PARPH-V8 and then to PAR-V8[2]. ***Note:*** P. ramorum *grows and sporulates better in the absence of hymexazol (i.e, on PAR-V8). It is best to subculture from suspect colonies early, before these colonies become over grown by faster-growing organisms.*

Figure 3. Characteristic structures of *Phytopthora ramorum* *(images courtesy of S. N. Jeffers, Clemson University)*



Second Baiting of Soil and/or Container Mix Samples

17. Remove composite samples from cold storage and hold at room temperature (22-24°C/72-75°F for 3 days to acclimate before baiting begins.
18. Bait samples again as described above in Steps 1-14.
19. After samples have been baited a second time, destroy or sterilize any remaining soil and/or container mix using an appropriate method (e.g., autoclaving).

[2] See Appendix B for recipe

Materials and Supplies

- Disposable gloves
- 4 L (1gallon) zip-to-close bags (at least 4 mm in thickness), avoid the bags with a "zipper" mechanism
- Trowel or other soil-sampling tool
- Wide bore soil tube (2.5 cm/1 in or larger)
- Permanent marker
- Insulated ice chest (with ice in bags or blue ice if external temperatures are above 21° C/70°F)
- Disinfesting solution (10% bleach, quarternary ammonium at the labeled rate or full-strength Lysol™ spray)
- Plastic or glass containers with lids; square, wide bottom containers work best (e.g., 0.5 L (1 pt) freezer boxes)(see Figure 2)
- Distilled water
- Single hole punchers, scissors or razor blades
- Forceps and scalpel
- 70% alcohol and flame for sterilizing laboratory utensils
- Paper towels
- Baits –Use rhododendron and/or camellia leaves that have been on the plant for at least one year; leaf pieces should be 5×5 mm squares (< 0.25 in) or 5 mm (< 0.25 in) dia. disks
- PARPH-V8 selective medium; 2 plates per baited container
- PAR-V8 selective medium (as needed for subcultures)

Growing Media Formulae

PARPH-V8 Selective Medium: For *Phytophthora* species *(Adapted from Jeffers and Martin, 1986; Ferguson and Jeffers, 1999)*

	Amount Per:	
Ingredient	**1.0 Liter**	**0.5 Liter**
Basal Medium		
Clarified V8 Concentrate*	50 ml	25 ml
Distilled Water	950 ml	475 ml
Difco Bacto Agar	15 g	7.5g
Amendments		
Delvocid [50% pimaricin]	10 mg	5 mg
Sodium Ampicillin	250 mg	125 mg
Rifamycin-SV [sodium salt]	10 mg	5 mg
Terraclor [75% PCNB]	66.7 mg	33.4 mg
Hymexazol	50 mg	25 mg

* Clarified V8 Concentrate is made from buffered V8 Juice (1.0 g $CaCO_3$/100 ml V8 Juice) clarified in one of three ways:

- centrifugation at 4000 RPM for 20 min followed by filtration using two layers of Whatman No. 1 filter paper under vacuum
- centrifugation at 7000 rpm for 10 min then filtration is not necessary
- vacuum filtration alone through a 1-2 cm deep layer of Celite

Clarified V8 should be frozen at -20°C in 50-ml aliquots (e.g., in disposable 50-ml centrifuge tubes).
PCNB and hymexazol are optional and can be omitted [e.g., to make PAR, PARP, & PARH]
• PCNB is useful to inhibit soilborne fungi on soil dilution plates
• Hymexazol inhibits **most** species of *Pythium* while allowing **most** species of *Phytophthora* to grow, although they may grow more slowly

Directions

1. Add ingredients for basal medium to a 2-L flask; thoroughly mix on a magnetic stirrer with a large stir bar in the flask
2. Autoclave for 20 min at 121°C and 15 psi; turn waterbath on to ~50°C
3. Add each amendment to a sterile water blank [5 ml distilled water in a 16-mm test tube]; vortex to mix
4. Cool medium in waterbath
5. Slowly stir medium with a magnetic stirrer in laminar flow hood
6. Vortex each amendment thoroughly and add to mixing basal medium
7. Use one additional sterile water blank to sequentially rinse all amendment tubes and then add rinse water to the medium; continue mixing medium
8. Pour plates relatively thin (i.e., about 15 ml/plate = 60 plates/liter); pour molten medium so it does not quite cover the entire plate; therefore, plates will need to be swirled gently to evenly distribute medium before it hardens
9. Cool plates at room temperature
10. Store plates inverted in plastic bags in the dark in a refrigerator
11. Plates should be used within 30 days

PAR-V8 Selective Medium: For *Phytophthora* species *(Adapted from Ferguson and Jeffers, 1999)*

Ingredient	Amount Per: 1.0 Liter	Amount Per: 0.5 Liter
Basal Medium		
Clarified V8 Concentrate*	50 ml	25 ml
Distilled Water	950 ml	475 ml
Difco Bacto Agar	15 g	7.5g
Amendments		
Delvocid [50% pimaricin]	10 mg	5 mg
Sodium Ampicillin	250 mg	125 mg
Rifamycin-SV [sodium salt]	10 mg	5 mg

* Clarified V8 Concentrate is made from buffered V8 Juice (1.0 g $CaCO_3$/100 ml V8 Juice) clarified in one of three ways:

- centrifugation at 4000 RPM for 20 min followed by filtration using two layers of Whatman No. 1 filter paper under vacuum
- centrifugation at 7000 rpm for 10 min then filtration is not necessary
- vacuum filtration alone through a 1-2 cm deep layer of Celite

Clarified V8 should be frozen at -20°C in 50-ml aliquots (e.g., in disposable 50-ml centrifuge tubes)

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References

Jeffers, S.N., and Martin, S.B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis. 70: 1038-1043.

Ferguson, A.J., and Jeffers, S.N. 1999. Detecting multiple species of *Phytophthora* in container mixes from ornamental crop nurseries. Plant Dis. 83: 1129-1136.