Phytophthora ramorum is a soil-borne plant pathogen well adapted to dispersal and movement via water. Described as a “water mold”, P. ramorum is more closely related to algae than fungi. For this reason, water samples collected from potentially infested nursery blocks can be tested for the presence of P. ramorum spores.

One of the following methods should be used to detect P. ramorum spores from water in nursery settings. Detection of P. ramorum by one of these methods indicates a problem exists, and triggers a more intensive survey.

1. **Bottle of Bait or ‘BOB’**. BOB relies on the use of host material for ‘baiting’ of collected water samples. After a water sample is collected in a one-liter bottle, host leaf pieces along with an intact host leaf are placed in the bottle. Bottles are incubated for three days, with the leaf pieces tested directly and the intact leaf incubated for symptom development. This is the preferred way of testing for Phytophthora spores in most nursery settings.

2. **Bait Bags or Bait Stations**. Bait bags containing rhododendron or camellia leaves are used to attract Phytophthora spores. This method is used for testing large holding ponds, lakes, streams and rivers. This method is most effective when water temperatures are between 46°F (8°C) and 71°F (22°C). After exposure, leaf baits are held briefly in a moist chamber to promote disease development and symptom expression.

**In vitro Water Sampling with Host Material Leaf Baits—Bottle of Bait (BOB) Technique**

**Bait Selection**
- Collect healthy leaves from a population of native or naturalized Rhododendron. Source host material must not have been sprayed with fungicide within the last three months. Avoid using newly acquired plants for this reason. Bait-source plants should be sufficiently large, robust and numerous enough to supply leaves during the entire duration of the survey.
- Use healthy leaves that have been on the plant for at least one year and are as free as possible from insect and mechanical damage. Do not use newly formed, succulent leaves. Present year leaf growth may be used after full leaf expansion and a period of hardening in summer.
- Place bait leaves in self-sealing plastic bags for refrigerated storage for no more than 14 days before use.

**Sample Collection**
- The number of water samples collected is based upon the number of water bodies present; availability of run-off water; and the overall size of the nursery. If this
sampling method is selected, 800-ml samples should be collected per sampling site. More samples will be needed for larger nurseries with more water and irrigation and drainage sources.

- Collect 800-ml of water from each sample point. Collect the cleanest sample possible by minimizing disturbance to the sediment, while avoiding plant and other floating debris. Use a 100-ml measuring cup, or disposable paper cup to fill a 1-liter screw cap plastic bottle in increments rather than filling the container all at once. If possible, collect the 800-ml sample from several collection points at each sample site.

- Record and mark the location of the sample site. If possible, GPS coordinates should be recorded for each sample, or a written description of the sample collection location should be recorded.

- Identification labels (time tape or masking tape) should be affixed both to the screw cap and the outside of each water collection bottle using a waterproof pen. Labels should be sufficiently coded to correspond with datasheet entries for each nursery and water body and should include date collected, water source (location), and nursery (i.e., nursery license number).

- When sampling drainage ditches or areas of moving water within nurseries, rinse bottles downstream with the water about to be sampled before water is collected.

- Each sample should be baited immediately with 20 freshly punched leaf pieces utilizing a hand held hole punch, and one whole, asymptomatic, non-wounded *Rhododendron* leaf. Sometimes, the punched pieces can become too degraded or have too many other organisms growing on them to successfully isolate *P. ramorum* if present. Because of this, the whole leaf is also used.

- Maintain collected water samples on their side in a cooler (without ice if external temperatures are cool or with enough ice to gradually cool water samples) for transport to the sample processing lab. Pack and ship water samples with rhododendron baits overnight mail with a cold pack or drive the samples directly to the laboratory.

- Maintain a record of the water sample information. Assign a unique sample number to each bottle.

Wash each 100-ml measuring cup with warm soapy water or use a new disposable cup between sample collections. Thoroughly and completely rinse each item. For best results, use an automatic dishwasher with a heated drying cycle or an autoclave to wash collection bottles. In most states, the laboratory will provide samplers with clean 1 L water collection bottles.

- Prepare enough cup/bottle sets for water collection at a number of sample sites per nursery. Use only clean, sanitized collection materials at each site and water source.
Sample Processing in receiving laboratory

- Bottles containing water samples and leaf baits are placed on their sides, and incubated for 3 days at 18 – 22°C in the dark.

- Following incubation, baits (leaf punches and whole leaf) should be removed, rinsed in distilled water, and blotted dry. Leaf pieces should be processed immediately for detection while whole leaves should be placed in moist chambers for up to 14 days to allow lesion development. In the unlikely event that no lesions develop, the sample can be considered negative and discarded.

- If symptoms appear on the incubated whole leaf, process the leaf using the same methods as for nursery leaf samples. Contact Mark Nakhla at: Mark.K.Nakhla@aphis.usda.gov for the latest version of the P. ramorum diagnostic protocols. The approved molecular diagnostic work instructions must be followed for these regulatory samples.

- Leaf pieces can be placed into selective PARPH-V8 medium for isolation of P. ramorum and then tested using approved molecular diagnostics, or they can be tested directly utilizing the PCR protocols. Leaf pieces which are to be tested directly can also be frozen and held for testing at a later time. The pieces from one sample can also be split with a number being tested through PCR and the remainder either going to plating and/or freezing.

- If symptoms appear on the incubated whole leaf, process the leaf using the same methods as for nursery leaf samples.

- Transfer the isolation plates immediately to a state or federally-approved processing laboratory via overnight courier, OR

- Maintain the agar plates at 20°C in the dark for at least three days.

- Check the plates at regular intervals under low magnification-using an inverted or dissecting microscope-for colonies with typical morphological characters of P. ramorum (e.g., coralloid hyphae, semi-papillate sporangia, and large chlamydospores).

- Contact laboratory personnel if P. ramorum is suspected from any samples.
Bottle of Bait Materials List

- 1-liter bottles per sample site (Nalgene wide mouth polypropylene provided by diagnostic laboratory).

- Multiple 100-ml plastic measuring cup or beaker and large capacity (100ml) syringe per water sample site. Disposable paper cups are also suitable for collecting water.

- Ice chest cooler (with a small amount of ice or other refrigerant if temperatures are warm outside. Isolate ice from sample with newspaper).

- *Rhododendron* leaves in plastic bag kept in a cooler until needed.

- Hand held paper hole punch (heart shape preferred).

Figure 1. Bottle of Bait (BoB) materials and leaf pieces plated into PARP media. Photos courtesy of Steve Oak - USDA Forest Service, Southern Region FHP, and Dr. Craig Webb, USDA-APHIS-PPQ.
In situ Water Sampling with Host Material Leaf Baits

Bait Selection

- Collect healthy leaves from a population of native or naturalized *Rhododendron*, *Camellia*, *Syringa* (lilac), or *Viburnum* spp. that have susceptible responses to *P. ramorum*. Source host material must not have been sprayed with fungicide within the last three months. Avoid using newly acquired host plants for this reason. Bait-source plants should be sufficiently large, robust and numerous enough to supply leaves during the entire duration of the survey.

- Use healthy leaves that have been on the plant for at least one year and are as free as possible from insect and mechanical damage. Do not use newly formed, succulent leaves. Present year leaf growth may be used after full leaf expansion and a period of hardening in summer.

- If bait leaves are smaller in size than 8 cm x 3 cm (3.2in x 1.2 in) at the widest point, use 8 leaves at each sampling location (one in each mesh bait bag). If leaves are larger than this dimension, four leaves per site can be used.

- Bait leaves may be wrapped in dry paper towels and sealed in self-sealing plastic bags for refrigerated storage for no more than 14 days before use. Dry towels help prevent leaves from breaking down.

- Place 4-8 leaves with the petioles (the stalk-like tissue that attaches the leaf to a stem) attached into each container (depending on leaf size, discussed above) at each sampling site. **Insert a uniquely numbered plastic tag into each bait bag.** The dates (date when bait was established and when bait was collected), water source (location), nursery information (i.e., nursery license number), tag number, water temperatures (initial temperature when baiting was established and final temperature when bait was collected) and GPS coordinate should be recorded on a data sheet.

Baiting Techniques

**Bait Bags**

Bait bags (approximately 12 inches by 12 inches) should be constructed of a durable, coarse nylon mesh material (e.g., non-wire window screening) and fastened together on three sides to allow sufficient overlapping material to seal bag edges (Figure 2). Single-use bait bags can be fashioned from muslin. Bait bags must have a separate pocket for each leaf, even if several small leaves are being used. This maximizes the surface area in the water. Exact configuration is not crucial and any bag type that can be closed and securely fastened (drawstring, flapped, rolled, etc.) is sufficient. Once leaves and numbered plastic tag are placed into the bag, it should be secured so the bait bag cannot float out and away.
• Firmly secure each bag by tethering to a stake driven into the ground or by suspending the bag from a rope that spans the width of the watercourse or pond. Bags should float near or just below the water’s surface for 7 to 14 days depending on water temperature (8 to 22°C). Baiting should be suspended when water temperatures exceed 22°C. If necessary, water can be tested using the BOB method. Bait bags should be placed in an area where water flows more slowly and pools. Locate the bags such that the leaves remain submerged even if water levels fluctuate. Do not leave the bait in the water for more than seven days if the water temperatures are at the higher range (22°C) as the leaf tissue will degrade and baiting efficacy will be sharply reduced.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>If = 22°C</td>
<td>Leave in water for 7 days</td>
</tr>
<tr>
<td>If ≥8°C, but &lt;22°C</td>
<td>Leave in water for 7 to 14 days</td>
</tr>
</tbody>
</table>

• When possible, shaded locations should be chosen.

• Record the water temperature.

• When placing bait bags in retention ponds, priority should be given to inflow and outflow points, preferably in shaded areas. A minimum of two bait bags per pond should be deployed.

**Bait Stations**

• An alternative to bait bags is a bait station, constructed of a PVC frame to which is attached ½ inch plastic fencing material to form an enclosure (see figures I-2 and I-3 below for photo and construction details). Leaves are attached with binder clips secured to the bottom of the enclosure. The numbered plastic tag is placed inside the enclosure before sealing.
• Attach the station to a stake driven into the ground or by suspending from a rope that spans the width of the watercourse or pond. The station should be deployed for at least 7 days (see Table I-1 above).

• When possible, shaded locations should be chosen.

• When placing bait stations in retention ponds, priority should be given to inflow and outflow points, located in shaded areas. A minimum of two bait stations per pond should be deployed.

**Bait Retrieval**

• After 7 to 14 days depending on water temperature (8 to 22°C), remove bait leaves (and the numbered tag) from each bag or station and rinse using water from the stream or pond thereby reducing the foreign matter (organic and soil particles) on the bait leaves.

• Wrap leaves in a moistened (damp, with no pooling water) paper towel and place in a 1 gallon self-sealing plastic bag. Be certain to place the numbered plastic tag from each bait bag into the plastic bag of the corresponding leaf bait tissues. Double bag the samples to prevent contamination or desiccation in the event a bag ruptures.

• Place all sample bags in an insulated cooler with blue ice or other sealed cooling media for transport to the laboratory. Do not place bait samples directly on the ice or cooling media; cardboard can be used to separate the ice from the bait samples.
Do not leave bags exposed to direct sunlight, or in hot conditions for an extended length of time before placing into a cooler. Clear plastic bags can solarize leaf bait samples if left in direct sunlight. Leaf bait samples can also be negatively affected if left in the hot interior of a truck or car if not placed in a cooler.

Record the date of bait retrieval as well as the water temperature at time of retrieval. This information should be entered into an appropriate database (see Appendix G of Nursery Survey Manual).

Following use, bait bags should be cleaned, followed by surface sterilization with either 95% ethanol or a 10% household bleach solution. To surface sterilize, bait bags should be either sprayed until runoff occurs or soaked. To ensure adequate time to sterilize and dry, wait at least 4 hours prior to reuse. Upon completing the cleaning process, rinse bags thoroughly with chlorinated tap or sterile water. Check for signs of deterioration and bag failure, replacing bags accordingly.

**Sample Transport, Storage, Shipping and Processing**

Samples should be kept in a cooler with a cooling source, or in a refrigerator until shipped. Do not permit the samples to freeze or dry out. Before shipping, contact laboratory personnel to coordinate sample reception and processing. Leaf bait samples should be packed just prior to shipping via overnight courier; do not prepare and store packed samples if they will not be shipped immediately.

Laboratory personnel should process bait samples using the same methods as for nursery leaf samples.

**Bait Station Construction Details**

**Frame**

- Frame is made using ½-inch-diameter PVC pipe and four elbow joints.
- Cut four lengths of PVC—two 11 ½ inch and two 10 ½ inch lengths.
- Glue pipes and joints into a rectangular shape using PVC primer and cement.

**Mesh**

The mesh used is a plastic, ½-inch fence material (with 3/8-inch openings) available at any national home improvement chain store.
- Dimensions are given both in inches and also based on number of squares (Figure 3)
- Mesh is secured to frame using plastic zip-ties.
- Leave one side of mesh half secured until leaves are inserted.
- Tie four small binder clips into mesh using plastic covered twist-ties; these clips hold leaves in place by petioles. This maintains separation between bait leaves, allowing for maximum water flow exposure for each bait tissue (Figure 4).
References


*Optional water sample protocol*

**Water Sampling for Filtration**

**Sample Collection**

Note: Samples should be processed within 8 hours of collection to optimize detection of Phytophthora spores. Samples will begin to degrade or decline as a detection tool after storage of 12 hours or more.

- Record and mark the location of the sample site. If possible, GPS coordinates should be recorded for each sample. Record the water temperature when feasible such as sample sites with ‘deep’ water such as retention ponds, large volume drainage areas.

- Identification labels (time tape or masking tape) should be affixed both to the screw cap and the outside of each water collection bottle using a waterproof pen. Labels should be sufficiently coded to correspond with datasheet entries for each nursery and water body and should include date collected, water source (location), and nursery (i.e., nursery license number).

- Before water is collected from a drainage area or moving water, rinse bottles downstream with the water about to be sampled.

- Maintain collected water samples in a cooler (without ice if external temperatures are cool or with enough ice to gradually cool water samples).

- Maintain a record of the water sample information, assign a unique sample number to each bottle.

- Between sample collection trips, wash each 100-ml measuring cup and 1-liter bottles with warm, soapy water. Thoroughly and completely rinse each item. For best results, use an automatic dishwasher with a heated drying cycle. Care should be taken when using soap and/or bleach as the residue could potentially affect the retrieval process.

- Prepare enough cup/bottle sets for water collection for a number of sample sites per nursery. Use only clean collection materials at each site and water source.

**Sample Processing**

- Most water samples can be vacuum filtered through polycarbonate membrane filters with 3-µm pores. Turbid or muddy water samples will need to be filtered through membrane filters with 5-µm pores. Typically a minimum of 10 plates and 10 filters will be required if 100 ml of water per filter are used. Surveyors should always take extra plates and filters to ensure that they have enough in the field.

- Place a filter funnel into a filter flask with a capacity of at least one liter and connect the flask to a vacuum source using plastic tubing; use a second filter flask as a trap between the flask with the funnel and the vacuum source (i.e., electric vacuum pump or
Appendix 7  
Water Sampling Protocol  
Updated March 22, 2014

- Wet filter holder with distilled water and place a polycarbonate membrane with the shiny side up or a polyvinylidene fluoride membrane with smoothest side up. Be sure the paper between the filters has been removed, the filter is aligned over the perforated area of the funnel, and that the filter is not wrinkled. Assemble the filter funnel and clamp it in place.

- Thoroughly mix the water sample by inverting the plastic bottle and/or swirling. Pour 100 ml of sample into the funnel. If the water is highly turbid, 100 ml may not be completely filtered by a single filter and smaller volumes should be used per filter to complete the sample. Conversely, if the water is extremely clear, additional water (up to 200 ml) may be processed by a single filter. More than one filter will be necessary to completely filter the 1 liter sample.

- Turn on the vacuum source initially at a low setting to filter water subsamples and adjust as necessary. The air should be turned off just prior to complete filtration of each subsample to avoid building up excessive vacuum pressure, which could damage Phytophthora spores.

- Rinse the inner surface of the funnel with distilled water to wash down any spores onto the filter which may be on the funnel wall. Apply the vacuum briefly to remove excess water.

- Gently remove sizable organic debris or soil particles trapped on the surface of the filter if it will prevent complete contact of the filter with the surface of PARPH-clarified V8 agar contained in Petri plates. Using forceps, gently lift the filter from the filter funnel and invert it so the collection side contacts the media surface. Smooth the filter with the forceps to remove air bubbles that may have formed between the filter and the agar media surface.

- Repeat the above steps until the entire 1 liter water sample has been filtered. A minimum of 10 agar plates per collection bottle should be produced resulting from filtration if 100 ml of sample is used per filter. Always have additional filters and plates prepared as the number required per sample can vary.

- Rinse the filter funnel assembly and forceps under hot, running tap water after each sample to avoid cross-contamination between samples. Do not disinfest the funnel with a bleach solution or alcohol as any residue may affect spore viability.

- Transfer the plates immediately to a state or federally-approved processing laboratory via overnight courier. Do not permit the samples to freeze or dry out at any time. Before shipping, contact laboratory personnel to coordinate sample reception and processing, OR

- Maintain the agar plates with filters at 20°C in the dark for at least three days.
Personnel should check agar plates after three days to see if any growth has occurred. If no growth is found, plates should be put back in the dark for an additional few days. *Phytophthora ramorum* grows slower than most other species of *Phytophthora* commonly found in water, leaving the filters on the agar plates for a three day incubation period is critical for recovery of this species.

- After the incubation period, remove the filters with sterile forceps and gently rinse the surface of the agar medium with running tap water to wash off small particles and bacterial colonies which may interfere with microscopic observation. Filters and rinse water should be treated and properly discarded to eliminate any risk associated with *P. ramorum*.

- Check the plates at regular intervals under low magnification-using an inverted or dissecting microscope-for colonies with typical morphological characteristics of *P. ramorum* (e.g., coralloid hyphae, semi-papillate sporangia, and large chlamydospores).

- Contact the appropriate hub laboratory personnel if *P. ramorum* is suspected from any samples.

Figure 5. Vacuum apparatus for water filtration and filter plated onto PAHRP-V8 plate. Photos courtesy of Dr. Steve Jeffers, Clemson University
Water Filtration Methods Materials List

- 1-liter bottles for sample collection (Nalgene preferred)
- 100-ml plastic measuring cup or beaker per sample site
- Thermometer (water-resistant type preferred)
- Ice chest cooler (with a small amount of ice or other refrigerant if temperatures are warm outside)
- Electric vacuum pump or hand-operated vacuum pump
- Sterile PARPH-clarified V8 selective medium (see protocol recipe) in disposable Petri plates; 10 plates per collection bottle; media plates can be stored in sealed plastic sleeves or bags in a refrigerator for two months before use
- 47-mm-diameter polycarbonate membrane filters with 3-µm pores (e.g., Sterlitech SKU No. PCT3047100 at http://www.sterlitech.com)
- 47-mm-diameter polyvinylidene fluoride (Durapore) membrane filters with 5-µm pores (e.g., Fisher Scientific #SVLP04700)
- Clamp-type filter funnel (Nalgene preferred)
- Two 1- or 2-liter filtering flasks (plastic or glass)
- Plastic tubing
- Bent-tip forceps
- Squeeze bottle containing distilled water
- Inverted or dissecting microscope