



Guidelines for Isolation by Culture and Morphological Identification of *Phytophthora ramorum*

Adapted from SOP 2/27/2003 of Nancy Osterbauer, OR Dept. Agriculture

April 21, 2004 (revised 6/5/07)

1. Laboratory Protocol

1.1 Log in samples by assigning each site a single identifying number or code (identifier). Record the State, identifier, the grower's license number (if applicable), the host(s), the inspector's initials as well as the location and date of inspection. It is suggested that samples be accompanied by a supplementary data sheet indicating the number of hosts present at each site. Save this data sheet in accordance with the NPDN format.

1.2 Identify the samples most symptomatic for *P. ramorum* infection for each host. ELISA can be utilized to pre-screen potentially symptomatic material for *Phytophthora* spp (see Appendix 5). Rinse the leaves in H₂O and then cut as described in Figure 1. If symptoms are not seen, or if an obvious lesion margin is not apparent, take samples from leaf tip, base or petiole. Change the H₂O between each site or between each subsample (host species or location) from the site. The rinse water must be sanitized or sterilized before disposal by autoclaving (see Section 3.2).

1.3 Label the PARP or PARP-V8 plate (Appendix 1) (near the edge) with the site's identifier and the date so sample identity is maintained.

1.4 Under a laminar flow hood, place one leaf section ("A" from Figure 1) onto the PARP or PARP-V8 plate. Slide the leaf section completely into the medium so that the leaf piece is placed near the bottom of the plate. This technique forces the pathogen to grow through the medium near the plate bottom, which allows for easy observation of growth using a compound microscope (100X) to view the underside of the plate. Place a maximum of 10 leaf sections into the medium of each plate. Do NOT place leaf sections from different nurseries or from different subsamples (hosts or locations) within a site on the same plate. Seal the plate with parafilm and incubate in the dark at room temperature (68-70°F) in a secure location. Examine for *Phytophthora* growth after 3-7 days.



Figure 1. Dashed lines indicate cuts. Place piece A into PARP medium. Label the PARP plate appropriately. Place the remainder of the leaf back into the original sample bag and place in a cold room for storage when finished sampling.

1.5 Identifying *Phytophthora ramorum* to species:

1.5.1. Examine the leaf tissues plated onto PARP plates with a compound microscope or a high-powered stereo (dissecting) scope. Turn the plate upside down and look for the large chlamydospores. Use the 10x magnifying lens on a compound scope or

the highest magnification of the stereoscope. A description of the fungus *in vitro* is given below in Appendix 2. *Phytophthora ramorum* will not form sporangia on PARP but may on PARP-V8. Record your findings on the data sheet and in the associated database. Also be aware that *Pythium* species and *Mortierella* species will also grow on PARP, but differ in culture morphology (*Pythium* spp. are very fast growing with cytoplasmic streaming often visible and *Mortierella* spp. tend to form wavy hyphae).

1.5.2. Transfer any *P. ramorum*-like isolates to a V8 agar plate (Appendix 1). Place one isolate per plate. Label the plate with the appropriate sample number. Seal the plate with parafilm and incubate at room temperature (68-70°F). Save the original PARP plate.

1.5.3. Allow the isolate to grow on V8 for 4 days. Cut out three 6-mm plugs of the isolate and place in a sterile petri dish, mycelium side up. Add enough sterile tap H₂O to the petri dish to be level with the top of the plugs but not covering the mycelium. Carefully wrap petri dishes with parafilm.

1.5.4. Place the plugs + H₂O in the dark at room temperature (68-70°F) or an incubator set at ≤ 18°C (64.5°F) and examine for sporangia after 48 hr. Sporangia will form on the edge(s) of the plug(s). Sporangia of *P. ramorum* are produced in clusters and deciduous and can often be seen in the water.

1.5.5. Determine whether the organism is *Phytophthora ramorum* using chlamydospore and sporangial characteristics (see Appendix 2).

1.5.6. Record findings. Isolates identified as *Phytophthora ramorum* must be confirmed by an official identifier. At present, the APHIS-NIS lab in Beltsville is the only official identifying site for culture morphology identification (Appendix 3). Suspect positive V-8 cultures should be forwarded as indicated in Appendix 3.

2. Quality Control Criteria

2.1 The current diagnostic protocol (as of 6/5/07) is that all samples that are ELISA positive and/or culture negative must be assayed using the validated nested PCR method by USDA or at authorized USDA laboratories. In other words, ELISA positive samples are extracted for DNA (see, http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/pcrprotocol4.pdf), and these DNA samples are forwarded to the NPGBL (which at present is the only authorized laboratory) as described in the protocol ([see Table 1](#)). If the diagnostic laboratory has not performed the ELISA test (but note that screening using ELISA is highly recommended), then culture negative samples need to have DNA extracted and these extracts sent to NPGBL for analysis (Dr. Laurene Levy, USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Biotechnology Laboratory (NPGBL), Building 580-East, Powdermill Road, Beltsville, MD 20705, 301.504.7100).

3. Safety and Disposal

3.1 Some of the components of PARP medium are **carcinogenic and extremely hazardous**. Laboratory personnel must follow OSHA's safety guidelines.

3.2 All plant samples, sample packaging material, and processed culture plates must be autoclaved at 121° C and 15 psi for 30 minutes prior to disposal.

3.3 All tools and other equipment must be sanitized and/or sterilized between samples.

Appendix 1: Media – Either PARP or PARP-V8 may be used in laboratory attempts to isolate *P. ramorum*. Cornmeal agar is no longer available from Difco so PARP-V8 has the advantage that no cornmeal agar is needed. Additionally, *P. ramorum* often sporulates on the PARP-V8 plates.

***Phytophthora*- selective media** NOTE: Some of the ingredients are carcinogenic and hazardous. Use caution when making these media

PARP-V8

Materials for basal medium

1. 50 ml clarified V8 juice (see below for preparation)
2. 15 g of Difco Bacto Agar
3. 950 ml of distilled water
4. Two liter flask or two liter autoclavable bottle

Materials for amendment of basal medium

1. 10 mg (0.010 g) of Delvocid Instant** (50% pimaricin) = 5 mg pimaricin
2. 250 mg (0.25g) of ampicillin sodium salt
3. 10 mg (0.1g) of rifamycin-SV sodium salt
4. 50 mg (0.05g) of PCNB (pentachloronitrobenzine) = 67 mg Terrachlor 75 WP
5. Four separate weighing boats or papers
6. Four clean 15-ml tubes
7. P1000 pipette and tips
8. sterile distilled water
9. tube rack to hold 15-ml tubes

** The current formulation of Delvocid Instant (DSM Food Specialties USA Inc, Menomonee Fall, WI 53051, 262-255-7955, 800-423-7906) uses a salt carrier whereas earlier formulations contained lactose. Either formulation can be used.

Materials for final prep of V8 PARP

1. 50-60 100 mm X 15mm petri dishes—15-20 ml of medium/dish

Preparation of clarified V8 juice:

In a clean beaker or flask, combine one or more cans of V8 Juice and 1 g of calcium carbonate (CaCO_3) for each 100 ml of V8 Juice used. Mix thoroughly on a magnetic stir plate. Dispense this mixture equally into centrifuge tubes or bottles. Centrifuge at 7000 rpm for 10 min; alternatively, centrifuge at 4000 rpm for 20 min and then filter through two layers of Whatman No. 1 filter paper. (To make a premeasured supply of V8: Dispense 50-ml aliquots of clarified, buffered V8 Juice into disposable, screw-capped 50-ml centrifuge tubes and store these in a non-frost-free freezer (-20°C). Tubes can be thawed at room temperature or in a microwave prior to use.)

Preparation of basal medium

Put 950ml of distilled water into an appropriate sized flask or bottle. Add 50ml of clarified V8 juice and 15g Difco Bacto Agar, and mix well (10-15 min on stir plate with low heat). If using flask cover mouth with aluminum foil, if using bottle make sure cap is very loose to prevent explosion in autoclave. Leave magnetic stir bar in flask. Autoclave basal medium for 35 min. with 10 min. exhaust. Place container of basal medium in water bath of 50-55C and allow to cool to this temperature.

Preparation of amendments

Don't prepare amendments until basal medium is cool and ready to be poured into petri dishes.

Do not filter sterilize amendments.

Weigh each of the four amendments in separate weighing boats or papers. Tap each into a labeled 15-mm tube with 5ml distilled water; vortex thoroughly to mix.

Final Prep of PARP V8

Have 50 to 60 100-mm X 15-mm petri dishes ready in hood. Once basal medium is cool, place flask or bottle on magnetic stirring plate and stir gently. Add amendments. The four amendments can be combined just prior to adding to basal medium. Use one additional sterile water blank to sequentially rinse all amendment tubes and then add rinse water to the medium; continue mixing medium. Under a hood with the blower running, pour a thin layer (15-20 ml) of medium into each petri dish. Put flask on stir plate frequently to keep PCNB in suspension. Invert dishes and place in a dark refrigerator or in an opaque container in the refrigerator (light degrades amendments). Best used within three weeks but can be stored for several months.

From AJ Ferguson and SN Jeffers. 1999. Detecting multiple species of *Phytophthora* in container mixes from ornamental crop nurseries. Plant Disease 83: 1129-1136.

PARP

Add 17g corn meal agar in 1000ml H₂O. Autoclave for 30 minutes (121° C/15 psi) then cool to 55°C. Once 55°C is reached add the following ingredients: 0.25g Ampicillin, 5ml PCNB solution, 1.0ml Rifampicin solution, 0.4ml Pimaricin (2.5% aqueous solution, Sigma Chemical cat. # P9703, P0440, or 80482)

The antibiotics in PARP are light sensitive. Plates should be poured under low-light conditions and stored in the dark. PARP medium can be stored for several months.

Penta-Chloro-Nitro-Benzene (PCNB) stock solution: Carefully dissolve 2 g PCNB crystals in 400 ml 95% ethanol heated in a 70°C water bath.

Rifampicin stock solution: 0.01 g rifampicin dissolved in 1.0 ml DMSO.

Note: We've received numerous questions about the possibility of using a different formulation of PARP than was provided in the SOD Protocol for isolation of *Phytophthora ramorum*. Making small variations such as using methanol in place of DMSO are probably fine. However, we strongly prefer that you use the formulation in the protocol for consistency across all labs. If your lab has routinely used a variation of PARP for isolating *Phytophthora*, and you have documentation that shows that it is just as effective as our formulation of PARP, please retain that documentation in your files in the event it is needed for future reference.

V-8 Juice agar medium: Add 2 g of CaCO₃ to 30 ml V8 and stir for 5-min. Centrifuge for 20-min at 4,000 rpm or allow to settle overnight. Save supernatant and discard solid waste. Dilute to 1 L with dH₂O. Add 15 g of granulated agar and autoclave for 20-min at 121°C. Cool and pour. (See [Preparation of clarified V8 juice](#) above for suggestions on stock clarified V8).

Appendix 2: Description of *Phytophthora ramorum* in vitro on V8 Plates

Colonies on V-8 plates in dark 24-30 mm in approximately 6 days, aerial mycelium whitish, appressed, with concentric rings (Fig. 2)

Chlamydospores (Fig. 3) numerous, thin-walled, globose, hyaline to pale brown, mostly 46-60 um, terminal or intercalary.

Sporangia (Fig. 4) ellipsoid, elongate-ovoid, caducous, often with a short pedicel, semipapillate, hyaline, 45-65 x 21-28 mm, single but usually in clusters.

Mortierella species are more like *Mucor* species, (Figure 5), but are also isolated on PARP. *Pythium* species could also be isolated on PARP, and will grow very quickly to cover a plate in 3-5 days. Cytoplasmic streaming is often observed in *Pythium* hyphae. These should not be transferred to V8 plates. Note that cultures can be mixed *P. ramorum*.

Appendix 3: Submission and Identification of Suspect Positive Samples

Laboratory personnel should send suspect positive V-8 cultures to Dr. Palm, USDA/APHIS/PPQ/National Identification Service at the address below. For transport, all culture plates should be wrapped with Parafilm. Pack the cultures in a self-sealing plastic bag and place that bag(s) within a second plastic bag. Place in a sturdy cardboard box or similar container so that it does not get damaged during shipping and handling. Samples must be accompanied by a PPQ Form 391 (see below) or equivalent information. Samples should also include copies of any datasheets. Send samples by overnight express shipping (Fed Ex, UPS) and label the package as "URGENT and FRAGILE." If you have any questions concerning the procedure, please email the labs before shipping.

Dr. Mary E. Palm, USDA/APHIS/PPQ
NIS Molecular Diagnostic Lab

B-580, BARC-East
 Powder Mill Rd.
 Beltsville MD 20705

Both morphological and molecular identification will be carried out by the NIS Molecular Diagnostic Lab. Dr. Palm will notify the PPQ National Program Manager, who will ensure that findings are communicated to the promptly to the appropriate Regional and Headquarters PPQ staffs and State contacts. When the identity is confirmed, the sample is considered to be POSITIVE.

Specimen Submission Form (PPQ Form 391) that should accompany specimens submitted to the USDA/APHIS National Identification Laboratories can be found in Appendix 4.

Appendix 4: PPQ Form 391.

PPQ Form 391. Specimen Submission Form (PPQ Form 391) to accompany specimens submitted to the USDA/APHIS National Identification Laboratories.

		This report is authorized by law (7 U.S.C. 147a). While you are not required to respond your cooperation is needed to make and accurate record of plant pest conditions.				FORM APPROVED			
						OMB NO. 0579-0010			
		U.S. DEPARTMENT OF AGRICULTURE		Instructions: Type or print information requested. Pres hard and print legibly when handwritten. Item 1 assign number for each collection beginning with the year, followed by collector's initials and collector's number. Example (collector, John J. Dingle); 83-JJD-001. <u>Pest Data Section</u> – Complete Items 14, 15 and 16 or 19 or 20 and 21 as applicable. Complete Items 17 and 18 if a trap was used.				FOR IIB/III USE	
		ANIMAL AND PLANT HEALTH INSPECTION SERVICE						Lot No.	
		SPECIMENS FOR DETERMINATION						PRIORITY	
		1. COLLECTION NUMBER		2. DATE		3. SUBMITTING AGENCY			
				MO DA YR		State PPQ Other			
SENDER AND ORIGIN	4. NAME OF SENDER			INTERCEPTION SITE	5. TYPE OF PROPERTY (<i>Farm, Feedmill, Nursery, etc.</i>)				
	6. ADDRESS OF SENDER				7. NAME AND ADDRESS OF PROPERTY OR OWNER				
	CITY, STATE ZIP				COUNTRY/COUNTY				
PURPOSE	8. REASON FOR IDENTIFICATION (“x” ALL Applicable Items)								
	A. Biological Control (Target Pest Name)			E. Livestock, Domestic Animal Pest					
	B. Damaging Crops/Plants			F. Possible Immigrant (Explain in Remarks)					
	C. Suspected Pest of Regulatory Concern (Explain in Remarks)			G. Survey (Explain in Remarks)					
	D. Stored Product Pest			H. Other (Explain in Remarks)					
	9. IF PROMPT OR URGENT IDENTIFICATION IS REQUESTED, PLEASE PROVIDE A BRIEF EXPLANATION UNDER “REMARKS”.								
HOST DATA		10. HOST INFORMATION				11. QUANTITY OF HOST			
		NAME OF HOST (<i>Scientific name when possible</i>)				NUMBER OF ACRES/PLANTS		PLANTS AFFECTED (<i>Insert figure & indicate number or percent</i>)	
								Number	
								Percent	
12. PLANT DISTRIBUTION		13. PLANT PARTS AFFECTED							
LIMITED		Leaves, Upper Surface		Trunk/Bark		Bulbs, Tubers, Corns		Seeds	
SCATTERED		Leaves, Lower Surface		Branches		Buds			
WIDESPREAD		Petiole		Growing Tips		Flowers			
		Stem		Roots		Fruits or Nuts			
PEST DATA		14. PEST DISTRIBUTION		15. INSECTS		NEMATODES		MOLLUSKS	
		FEW		NUMBER SUBMITTED		LARVAE PUPAE ADULTS		CAST SKINS EGGS NYMPHS JUVS. CYSTS	

	ABUNDANT									
	EXTREME									
16. SAMPLING METHOD			17. TYPE OF TRAP AND LURE				18. TRAP NUMBER			
19. PLANT PATHOLOGY – PLANT SYMPTOMS (“X” one and describe symptoms)										
	ISOLATED	GENERAL								
20. WEED DENSITY					21. WEED GROWTH STAGE					
FEW	SPOTTY	GENERAL	SEEDLING	VEGETATIVE	FLOWERING/FRUITING	MATURE				
22. REMARKS										
23. TENTATIVE DETERMINATION										
24. DETERMINATION AND NOTES (Not for Field Use)									FOR IIB/I USE	
									DATE RECEIVED	
									NO.	
									LABEL	
									SORTED	
									PREPARED	
									DATE ACCEPTED	
SIGNATURE						DATE			RR	

PPQ FORM 391 Previous editions are obsolete.

(JUL 86)



Fig. 2. Fifteen day old culture of *P. ramorum* on V-8.



Fig. 3. Chlamydospores of *P. ramorum* (DIC optics).

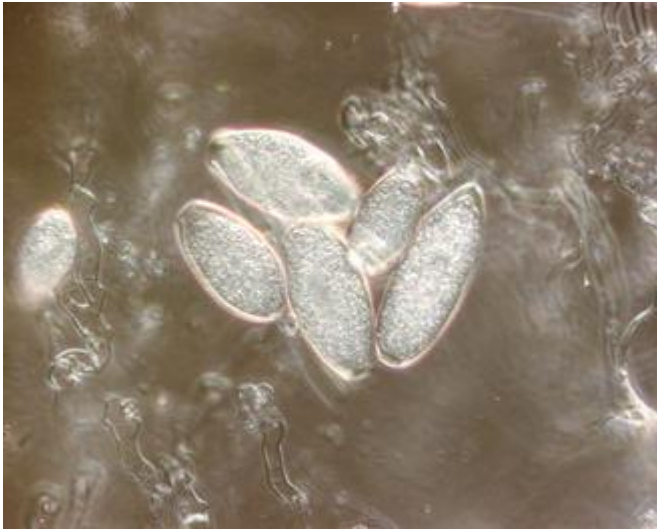


Fig. 4. Semi-papillate sporangia of *P. ramorum* (DIC optics)

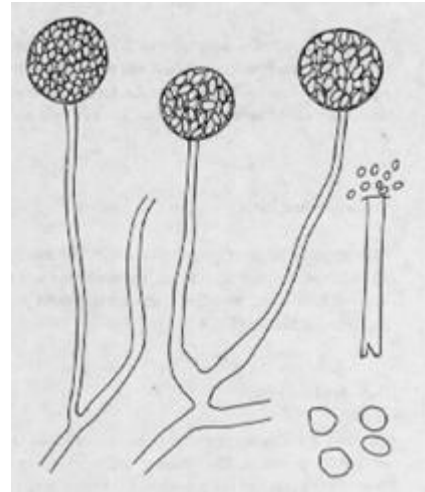


Fig. 5. Examples of *Mortierella* species

(<http://www.botany.utoronto.ca/researchlabs/mallochlab/malloch/Moulds/Mortierella.html> and <http://www2.truman.edu/~jherrera/Zygomycetes/Mortierella/Mortierella.html>)