Methods for isolation, maintenance, sporulation, and storage of *Phytophthora species*

In this protocol, we present the strategies for isolation, maintenance, sporulation, and storage of *Phytophthora* isolates. This SOP, along with other related SOPs, can be found on the Morphology page of *IDphy: Molecular and morphological identification of Phytophthora based on the types*.

I. Related SOPs

SOP-PID-07.00 Morphology: isolation and methods for production of asexual and sexual phases.

II. Introduction

Various media amended with antibiotics allow the isolation of *Phytophthora* species from infected plant material including roots, stems, leaves, trunks, or fruits. This SOP provides guidance for isolations from plant material, maintenance of cultures, and induction of sporulation to aid in morphological identification. For additional information and references on this topic, please refer to the references section and our publication (Martin F.N., Abad Z.G., Balci Y. & Ivors K. 2012. Identification and detection of *Phytophthora*: reviewing our progress, identifying our needs. Phytopathology 96: 1080-1103).

III. Isolation from host tissue

Materials

- Two petri dishes with selective culture media/sample: cornmeal agar (CMA) + P_{10}ARPH, or CMA + P_{10}VPH. Carrot agar, baby lima bean agar, or V-8 agar (clarified or not) plus antibiotics can also be used for the

original isolation of species of *Phytophthora*. Some species are also isolated in PARP media without the addition of hymexazol. See pages 3 to 6 for information on selective culture media with antibiotics.

- Scalpel, knife, razor, tweezers, scissors, paper towel, marker, 70% alcohol, distilled deionized sterile water, stereoscope, compound microscope

Selecting the tissue

- For isolation from roots, depending on the symptoms, take: individual symptomatic roots (with part of the lesion and part of healthy tissue), or a group of symptomatic roots. Observe disease symptoms and signs, including small discolored or necrotic areas, root tips, and general death of the root system. Injuries in the root system can be reflected in symptoms in the aerial part of plants, such as yellowing, stunting, or wilting.
- For isolation from leaves, crowns, stems, and storage organs, it is important to select tissue from the margin of diseased and healthy tissue (from newly infected rather than dead tissue).

Procedure

1. Wash the tissue under tap water to release soil, debris, and external contamination.
2. Dry the tissue in a paper towel.
3. Cut roots into small pieces, leaving them drying on the paper towel.
4. Using tweezers, place samples in 6 points of the plate in at least two petri dishes containing selective media (i.e. Phytophthora P_{10}ARPH). Gently press tissue into the media using the tweezers. Some samples of root rot require transfer into two or more different selective media.

5. Incubate plates in the dark at room temperature (25ºC) for 24-48 hours.
6. Observe under compound microscope, inverting plates.
7. Observe the area close to the tissue pieces for mycelial growth (i.e. coralloid coenocytic mycelia for *Phytophthora* species). Mark a circle on the plate at the edge of colony, selecting **hyphal tips** (if possible).
8. Transfer marked areas to new selective media (i.e. P_{10}ARPH), and after 2-3 days transfer again to general media.
9. In many instances, when fruiting bodies are produced (i.e. sporangia, oospores, etc.), the organism associated with the disease can be identified. Other cases require future transfers and additional procedures for identification.

Notes

- Be aware other groups of plant pathogens (fungi, bacteria, nematodes, phytoplasmas, viruses) or abiotic diseases can cause symptoms on plants similar to those caused by *Phytophthora*.
- To prevent contamination, use clean tweezers, scalpels, or razors. You can sterilize tweezers and scalpels by submerging in 70% alcohol, allowing the alcohol to evaporate, or by flaming. Remember to sterilize instruments every time you work with different samples.
- Production of sporangia is favored by light, but antibiotics are light-sensitive, so incubate samples in selective media in the dark.
- Compare disease symptoms and signs to the information and images in reference books and those posted on the Internet.

IV. Pancake method to ensure pure cultures

The technique developed by Sleeth (1945) for isolation of *Pythium* species free from bacterial contamination is also useful for the culture of *Phytophthora* species.

- At the center of a sterile empty petri dish, place a piece of agar containing the isolate.
- Using a sterile spatula, cut media into quarters and remove a block of agar (large enough to cover the piece with the isolate) from a selective agar plate (PARP, P10VP, etc.) and place it on top, covering the isolate sample.
- Incubate at room temperature for 2-3 days. Observe under the microscope that the hyphae have grown through the media and into the surface of the block of agar.
- Using a sterile scalpel, remove a thin layer from the surface of the media containing the uncontaminated hyphal tip (if possible).
- Transfer the plug onto nutrient agar to make sure that the sample is free of bacterial contamination.

V. Selective media for isolation of *Phytophthora*

1) **PARPH Medium**[a] (Kannwischer and Mitchell 1974, with modifications by the Plant Disease and Insect Clinic, North Carolina State University). This recipe is recommended for the original isolation of *Phytophthora* as well for other oomycetes and for *Phytopythium* and *Pythium* without hymexazol.

   Prepare 400 ml of cornmeal agar base (6.8 g/400 ml H_2O or 17 g/L H_2O), autoclave, and store in 500 ml bottles. Prepare antibiotics in stock solutions, see below for recipes. At the time of use, when agar is melted and cooled to about 45-50ºC, add antibiotic suspensions just before plates are poured, as follows:

   - Add 1 (one) vial containing the stock of ampicillin, hymexazol, and PCNB.
   - Add 8 drops pimaricin.
   - Add 8 drops rifampicin.

   Antibiotic suspensions and solutions:

P_{10}ARP	**Amt/liter**	
pimaricin	10 mg	(Delvocid instant 50% a. i., Gist-brocades Food Ingredients, Inc. N93 W14560 Whittaker Way, Menomonee Falls, WI 53051. Phone: 414-255-7955)
ampicillin	250 mg	(Sigma Chemical Co. # A-9518 sodium salt >98% a.i.)
rifampicin	10 mg	(rifampicin SV, sodium salt 100% a.i., Sigma Chemical Co., # R-8626)
PCNB	100 mg	(pentachloronitrobenzene, Terraclor 75%, Olin Mathieson Chemical Corp., Little Rock, AK 72203)

   Modifications

   - P_5ARP: reduce pimaricin to 5 mg/L
   - P_{10}ARPH: Add hymexazol (Tachigaren 70%, only available from a Japanese company at 50 mg (a.i.)/liter. This reduces growth of *Pythium* and *Mortierella.* Hymexazol inhibits the growth of *P. cactorum, P. infestans, P. megasperma, P. phaseoli, P. porri,* and *P. syringae* (Erwin and Ribeiro 1994).
   - Increase the PCNB concentration to 125 mg (a.i.)/liter.

Stock solutions

a) **The ampicillin, hymexazol, and PCNB** are made into solution in sterile water together, frozen in Falcon tubes (15 ml vials), and added to plates when poured.

		Amounts for 100 ml suspension
ampicillin	25 mg/ml of suspension	2.5 g
hymexazol	5 mg/ml of suspension	0.500 g
PCNB	12.5 mg/ml of suspension	
Terraclor is 75% a.i, so use 16.7 mg/ml suspension		1.670 g

Freeze suspension in 5 ml units. Add one vial to 400 ml bottle of cornmeal agar just before pouring.

b) **Pimaricin** is placed into suspension in sterile water. Store in refrigerator in amber bottles.
Add 2 g Delvocid to 100 ml H_20 (2g/100 ml = 2000 mg/100 ml = 20 mg/ml)
1 ml of concentrate will contain 20 mg Delvocid (10mg a.i. pimaricin) = 10 ppm
Add 8 drops pimaricin / 400 ml cornmeal agar.

c) **Rifampicin** is prepared as a solution in 95% ethanol. Store in refrigerator in amber bottles.
Add 1 g rifampicin to 100 ml solvent (1 g/100 ml = 1000 mg/100 ml = 10 mg/ml = 10 ppm)
Add 8 drops rifampicin / 400 ml cornmeal agar.

2) P_{10}VP/H agar (Tsao and Ocano 1969)

Prepare 400 ml of cornmeal agar base (6.8 g/400 ml H_2O or 17 g/L H_2O), autoclave, and store in 500 ml bottles. Prepare antibiotics in stock solution, see below for recipe. At the time of use, when agar is melted and cooled to about 45-50°C, add antibiotic suspensions just before plates are poured.

Stock solution

Into 100 ml sterile distilled H_2O in sterile bottle, place:
1.0 g vancomycin (pure)
0.1 g pimaricin (50%)
0.53 g PCNB (95%)
0.25 g hymexazol* (99%)

*If you do not add hymexazol, the medium will be selective for all pythiaceous fungi. Shake well to dissolve/suspend. Store refrigerated away from light.

VI. General culture media

COFFEY'S PEA MEDIUM (BROTH OR AGAR). This media is excellent for the growth of many species of *Phytophthora* including *P. infestans* and other related species.

Recipe for 1500 ml

- Place ~450 g frozen petite peas in ~900 ml distilled water.
- Boil in microwave for 12-15 min.
- Pass through coarse sieve and discard peas.
- Add:

12 g	sucrose (i.e. Sigma)
3 g	L-asparagine (i.e. Sigma)
1.5 g	L-methionine (i.e. Sigma)
150 mg	β-sitosterol (optional) (i.e. Sigma)

- Add distilled water to bring volume to 1.5 L (1500 ml).
- For agar medium add 7.5 g Difco Bacto agar to each bottle of 500 ml of medium.
- Autoclave for 40 minutes.

BABY LIMA BEAN AGAR (for *Phytophthora* species)
Add 50 g of baby lima beans to 500 ml of distilled deionized (DD) H_2O, and autoclave for 5 min. Filter through 4 layers of cheesecloth, and discard the lima beans. Add DD H_2O for a total volume of 1 L, then add 17 g agar.
Autoclave 15 min at 121ºC.

This medium is excellent for oospore production in many *Phytophthora* species. See sporulation techniques below for oospore production in heterothallic species.

CORNMEAL AGAR (CMA) (for *Phytophthora*, *Pythium,* and other oomycetes)
Add 17 g Difco cornmeal agar to 1 L of DD H_2O.
Autoclave 15 min at 121ºC.

OATMEAL AGAR (for *Phytophthora*, *Pythium,* and many fungi)
Boil 80 g of oatmeal in 700 ml of DD H_2O for 15 min, strain through cheesecloth, and add 15 g of agar.
Autoclave 15 min at 121ºC.

CARROT AGAR (for *Phytophthora* species)
Add 50 g of baby carrots to 500 ml of DD H2O, and autoclave for 5 min. Filter through 4 layers of cheesecloth, and discard the solids. Add DD H_2O for a total volume of 1 L, then add 17 g agar.
Autoclave 15 min at 121ºC.

This medium is good for oospore production in many *Phytophthora* species, also for colony morphology and temperature analysis.

V-8 JUICE AGAR (for *Phytophthora*, *Pythium,* and many fungi)
Mix 200 ml V-8 juice with 2.5 g $CaCO_3$. Centrifuge to clarify (5 min at 2,000 rpm), decant, and discard pellet. Add 18 g agar, and then add DD H_2O for a total volume of 1 L.
Autoclave 15 min at 121ºC.

VII. Techniques for asexual phase sporulation

Soil stock solution (1 L)

- Add 1 L of DD H_2O to 100 g of soil, stir soil mixture with a magnetic stirrer for 1 minute.
- Leave to settle for 5-10 minutes, decant upper portion through three layers of cheesecloth to remove organic debris.
- Allow suspension to settle overnight.
- Filter upper part through EITHER:
    - a Millipore Stericup and Steritop filter unit / Nalgene 75/90mm filtration unit [with vacuum], OR
    - One layer of Whatman #1 filter paper [with Buchman funnel and vacuum].

  Note: both filtration units (Stericup/Steritop and Nalgene) and filter paper would have to be changed periodically to ensure efficient filtering.
- Dispense solution into 500 ml bottles and store in the refrigerator. Note: final solution should be free of debris/organic matter and have a slight yellow color.

10% sterile soil solution (1 L)

- Add 900 ml of DD H_2O to 100 ml of the full-strength soil stock solution. Note: final solution should be colorless.
- Dispense solution into 250 ml bottles and autoclave for 20 minutes.
- Store at room temperature.

Sporangia production for *Phytophthora* species

Most cultures of *Phytophthora* species can often be stimulated to form sporangia by transferring small plugs of active cultures growing for 3-7 days in pea agar, 5% V-8 juice agar, lima bean agar, or cornmeal agar from the border of colonies into water blank cultures. Using an empty plate, deposit a few blocks from the culture, and add water to the level of agar. 10% soil solution or rainwater can be used instead of distilled deionized H_2O to stimulate sporangia production (see Tables A and B below). Do not overflow the culture. Incubate under continuous fluorescent light for 24-48 hrs for sporangia production. Sporangia may be observed at the margin of the colony. Hyphal swellings and chlamydospores are also often produced using this method. One excellent way to induce sporangia production and zoospore release for most species is using mats of mycelia under water blanks or soil solution exposed to continuous light for 24-48 hrs. These mycelia are first grown in pea broth after being scraped from cultures on pea agar, V-8 agar, or carrot potato agar.

Production of asexual stage (sporangia) of different *Phytophthora* species

Table A, sporulation in water of agar disks of isolates growing in different culture media

Sporulation: agar discs in water

		Pea A		PDA		V8A		LBA	
Species	**Isolates**	spor.	zoos.	spor.	zoos.	spor.	zoos.	spor.	zoos.
P. cactorum	P0714_SS#9	+++	+++	–	–	+	+	+	–
P. capsici	P3605_ET#33	++	+	–	–	+	+	+	–
P. cinnomomi	P2110_ET#12	–	–	–	–	–	–	–	–
P. citricola	P0716_ET#34	+++	+++	–	–	+	+	+	+
P. cryptogea	P1738_ET#16	–	–	–	–	–	–	–	–
P. citrophthora	P0479_#60	+++	++	+	–	+	+	+	+
P. drechsleri	P1087_NT#17	+	+	–	–	+	+	–	–
P. heveae	P3428_T#67	++	++	+	+	+	+	+	+
P. meadii	P29007_LTT#81A1	+++	+++	++	–	++	+	+	–
P. nicotianae	P7661_NT#44	+++	+++	++	–	++	+	++	+
P. palmivora	P0255_SS#46	++	+	+	–	+	+	+	+
P. vignae	P3019_NT#30	–	–	–	–	–	–	–	–

Synchronize zoospore releasing

Table B, sporulation in different kinds of liquid cultures.

Sporulation: different water

		Distilled water		Sterile soil solution		Soil solution		Sterile rain water		Rain water	
Species	**Isolates**	spor.	zoos.	spor.	zoos.	spor.	zoos.	spor.	zoos.	spor.	zoos.
P. cactorum	P0714_SS#9	++	++	+++	+++	+	+	++	++	++	++
P. cinnamomi	P2110_ET#12	–	–	–	–	–	–	–	–	–	–
P. citricola	P0716_ET#34	++	++	+++	+++	+	+	++	++	++	++
P. drechsleri	P1087_NT#17	+	–	+	+	+	+	–	–	–	–
P. nicotianae	P7661_NT#44	++	++	+++	+++	++	++	++	++	++	++
P. palmivora	P0255_SS#46	+	+	++	++	+	+	++	++	++	++
unknown	TARI 90173 (A01)	–	–	+	+	+	+	+	+	+	+
unknown	TARI 212112 (B01)	++	++	+++	+++	+++	+++	++	++	++	++
unknown	TARI 90125 (C01)	–	–	–	–	–	–	–	–	–	–
unknown	TARI 97014 (D01)	++	++	+++	+++	+++	+++	++	++	++	++
unknown	TARI 208047 (E01)	++	++	+++	+++	+++	+++	++	++	++	++

Tables courtesy of Dr. Jin-Hsing Huang from Taiwan Agricultural Research Institute (TARI)

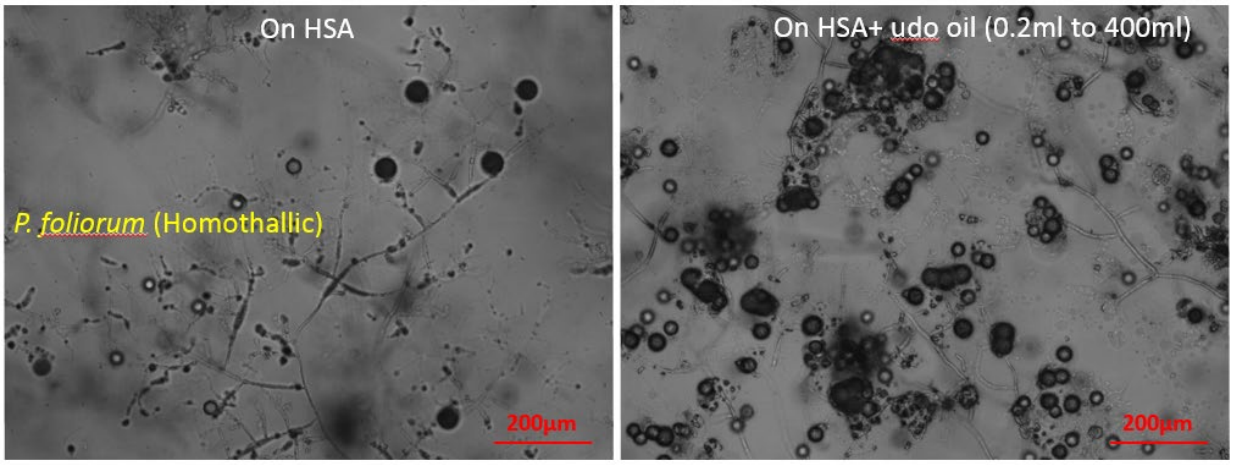
Zoospore release

For many species of *Phytophthora, Phytopythium,* and *Pythium*, sporangia can be stimulated to release zoospores by chilling the culture (15 min-2 hr), then placing it at room temperature (1-3 min). Repeat the process until zoospores are produced. Try using 2 or 3 plates at 5ºC for different times to see what works best.

VIII. Techniques for sexual phase sporulation

Oospores of homothallic species are produced in hemp seed agar, lima bean agar, baby carrot agar, and malt extract agar. Production of oospores in heterothallic species requires the presence of the strain with the opposite compatibility type (A1 – A2). The addition of sterols (i.e. β-sitosterol), and fatty acids are used to stimulate oospore production in *Phytophthora* species.

For pairings of heterothallic species we recommend the use of hemp seed agar with omega- 3, 6, 9 fatty acids (alpha-linolenic, linoleic, gamma-linoleic, and oleic) in the product Udo's Oil (made with organic flax, sesame and sunflower seed oils, manufactured by: Flora, INC, Lynden, WA, USA) at concentration of 200 µl/400 ml media. Plates with pairings are incubated in the dark at 20ºC. Macadamia oil also works very well for the production of oospores, compared to other oils.



Left. Production of oospores of *Phytophthora foliorum* (homothallic species) growing for 7 days on hemp seed agar (HSA). **Right.** The same species growing in HSA plus Udo's Oil. Similar results were observed when Macadamia Oil was added into the culture media. Images courtesy of Dr. Jin-Hsing Huang from Taiwan Agricultural Research Institute (TARI).

IX. MAINTENANCE AND STORAGE OF CULTURES

Numerous references exist on the short-, medium-, and long-term maintenance and storage of *Phytophthora* cultures (Erwin and Ribeiro, 1996). The most commonly used methods for long-term storage are liquid nitrogen or ultra-cold conditions (-80ºC). One easy and effective technique for *Phytophthora* is the storage of agar plugs immersed in sterile water in plastic vials. All species can be simply stored on lima bean or cornmeal agar disks in sterile water with sterile hemp seed for long-term storage (10-12ºC). More elaborate and expensive cryo-storage in liquid nitrogen can is also possible (Tooley, 1988). The following system for storage was provided by Paul Tooley (USDA-ARS) in January 2018.

Rye vials for storage of *Phytophthora* species

Place 4-8 grains of whole grain rye (available at health food stores) into about 10 ml of distilled water in a 20 ml Wheaton product 225288 vial and autoclave. (Recommended: Wheaton Science Products 225288 clear borosilicate glass sample vial in lab file with 14B rubber lined cap, 20 ml capacity.) When cool, store refrigerated until use.

To use, place several small agar pieces containing *Phytophthora* sp. into the vial. Let grow for about a week (we grow at 18-20ºC in the dark) until you see mycelia growing out from around the seeds and up into the water. Then tighten the cap, and (optional) parafilm around the cap to prevent evaporation if they are to be stored for a long time. Store at 18-20ºC for up to 2 years. Some will even go longer than that, depending on the species.

To recover the *Phytophthora* from the vial, the seeds/mycelium tends to make one large clump, so reach down into the vial with a long-pronged sterile forceps, and also have a sterile scalpel or other tool in your other hand to tease apart the mass of seed/hyphae so you can get out a single seed for plating. Or, you can remove the entire clump into a sterile Petri dish, for example, and then tease it apart.

Phytophthora culture growing in rye seed vial (Paul Tooley, Ph.D. USDA-ARS)

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