Identifying Species of *Phytophthora*

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The first and most important step to identify isolates of *Phytophthora* spp. is to have a clean culture consisting of a single isolate (i.e., one genotype). Usually this is achieved by using appropriate selective media for isolation and then transferring a portion of a single hypha to establish the culture in question.

Observe and record all morphological characters associated with any unknown isolate; you never know what might be a "key" character. In general, three basic morphological criteria are needed to identify species of *Phytophthora* on a regular basis:

- colony morphology on standard media,
- sporangium characteristics, and
- oospore characteristics.

Usually other structures will be produced (if the unknown produces other structures…) while cultures are manipulated to obtain these criteria—like hyphal swellings, chlamydospores, characteristic hyphae, etc. Occasionally, additional information will be necessary to differentiate certain species—like cardinal temperatures for growth, utilization of specific nutrients or compounds, growth on a specific medium, etc. These will be specified in the keys being used.

Colony Morphology

Observe colony morphology on one of the selective media (e.g., PAR, PARP, PARPH) and on a nutrient limited medium without antimicrobial amendments (e.g.., CMA, cV8A, scV8A, etc.). Be consistent with the media used so patterns will become "standardized".

Characters to note:

- mycelium growth habit—aerial or appressed
- mycelium pattern—uniform, radiate, stellate, petalloid/rosette, other??
- structures present in agar—sporangia, oospores, hyphal swellings, chlamydospores

Sporangium Production

For most species, sporangia form most consistently in liquid and in the presence of light. Therefore, agar plugs of young, actively-growing mycelium are flooded with a liquid and these flooded plugs are placed under light to stimulate sporangium production. Although many different liquids have been used over the years, I have found non-sterile soil extract solution (i.e., NS-SES) works well for all species examined to date and is relatively easy to make. Regular V8A (unclarified, 10%) or carrot agar works well as the agar medium.

Sporangium production procedure:

- transfer unknown to V8A and
- incubate the culture in the dark at 20-25°C
- while the mycelium is young and actively-growing (i.e., colony diameter one-half of petri dish diameter), aseptically remove agar plugs (3-5 mm in diameter) from the colony periphery ; cork borer sizes 1-3 work well…
- place 5-10 plugs in a small (60-mm diameter) petri dish
- flood plugs with just enough NS-SES to completely cover all plugs (usually 7-10 ml); too much liquid will inhibit sporangium formation
- Place petri dishes under continuous fluorescent lights at room temperature (22-25°C) or in an incubator at 20-25°C
- examine plugs after 12-24 hours; look for the first flush of mature sporangia
- if sporangia have not formed at 24 hours, leave plugs under lights for another 24 hours
- if sporangia still do not form, remove NS-SES with a pipette, add a similar amount of distilled water, place plugs back under lights for 24-48 hours
- once sporangia have formed, remove from liquid and fix and stain sporangia (see below)

Oospore Production

Oospores usually are formed in the dark and at a temperature lower than the optimum temperature for growth. Therefore, I routinely incubate cultures in a dark incubator at 20°C. Sterols are needed in the medium for oospore production, but many undefined, complex media (e.g., CMA, V8A, etc.) contain sterols in some amount. Clarified V8A (i.e., cV8A) and carrot agar (i.e., CA) are considered to be among the best media for oospore production. I prefer "Super" cV8A—which is 10% V8A amended with ß-sitosterol, thiamine, and other ingredients to enhance oospore formation. Usually oogonia and antheridia will be present in 7 days and oospores will be present in 14 days—if they are going to form. However, it is recommended to leave plates for 4-6 weeks to give oospores ample opportunity to form with "uncooperative" isolates. Isolates that do not form oospores in single-strain culture (i.e., are not homothallic), probably are heterothallic and need to be crossed with a compatible mating type.

Oospore production procedure:

- transfer unknown to a plate of Super cV8A; use either 60- or 90-mm diameter plates
- incubate cultures at 20°C in the dark
- examine weekly for up to 6 weeks
- when oospores have formed and are mature (i.e., oospore wall is thick and fully developed)—remove small agar blocks or plugs (3-5 mm in diameter) and fix and stain as described below

Fixing and Staining Structures in/on Agar Plugs or Blocks

- Prepare a solution of 0.05-0.1% acid fuchsin in full strength (85%) lactic acid
- immerse plugs in this solution overnight; the acid will dissolve the agar and fix the mycelium
- mount mycelium mats in clear lactoglycerol for microscopic examination
- measure and record at least 25 structures of each type

Non-Sterile Soil Extract Solution (1.5%)

Reference: Jeffers, S. N., and H. S. Aldwinckle. 1987. Phytopathology 77:1475-1482.

Ingredient	Amount/Liter	Amount/500 ml
Field soil	15 g	7.5 g
Distilled water	1000 ml	500 ml

Procedure

1. Collect field soil [preferably a loam or silt loam] from an area where plants are growing; *Phytophthora* should ***not*** be present in this soil.
2. Mix soil and water in a flask with a magnetic stirrer for at least 4 hr.
3. Allow suspension to passively settle for at least 4 hr or, preferably, overnight.
4. Decant supernatant and centrifuge in 250- or 500-ml Nalgene bottles for 10 min at 8000 rpm; this is a force of approximately 10,000 *g*
5. Filter through 2 layers of Whatman No. 1 filter paper [with vacuum]; this removes floating organic debris.
6. Store NS-SES in a glass bottle in the refrigerator [at approximately 4 C].
7. NS-SES can be used for several months although it is best when "fresh"; although, recently we have discovered it needs to "age" for several days to a week before it works most effectively…
8. This solution can be sterilized by filtration or autoclaving if necessary–i.e., S-SES; sterile SES is not as effective as nonsterile SES at stimulating sporangium formation

PAR(PH)-V8 Selective Medium: For *Phytophthora* species

References: Jeffers & Martin. 1986. Plant Disease 70:1038-1043
Ferguson & Jeffers. 1999. Plant Disease 83:1129-1136

***Note:** **This medium is much better than its CMA-based counterpart because V8 stimulates spore production by *Phytophthora* spp. on isolation plates—see paper by Ferguson & Jeffers…**

Ingredient	Amount per 1.0 Liter	Amount per 0.5 Liter	A. I. Concentration (PPM)
Basal Medium			
*Clarified V8 Concentrate	50 ml	25 ml	
Distilled Water	950 ml	475 ml	
Difco Bacto Agar	15 g	7.5 g	
Amendments			
Delvocid [50% pimaricin]	10 mg = 0.01 g	5 mg	5
Sodium Ampicillin	250 mg = 0.25 g	125 mg	~250
Rifamycin-SV [sodium salt]	10 mg = 0.01 g	5 mg	~10
**Terraclor [75% PCNB]	66.7 mg = 0.0667	33.4 mg	50
**Hymexazol	50 mg = 0.05	25 mg	50
or **Tachigaren [70% Hymexazol]	71.4 mg = 0.071 g	35.7 mg	50

* Clarified V8 Concentrate = Buffered V8 Juice [1.0 g CaCO3/100 ml V8 Juice] clarified by:
- centrifugation [4000 RPM for 20 min] & filtration [2 layers of Whatman No. 1 with vacuum], or
- spin @ 7000 rpm for 10 min then filtration is not necessary

then, freeze 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 particularly useful to inhibit soilborne fungi on soil dilution plates
- Hymexazol inhibits **most** *Pythium* spp. while allowing **most** *Phytophthora* spp. to grow

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. Best if plates are used within several weeks—but they will keep for months

S. N. Jeffers ***Clemson University*** ***Revised 30 March 2007***

PAR(PH)-CMA Selective Medium: For *Phytophthora* species

References: Jeffers & Martin. 1986. Plant Disease 70:1038-1043
Ferguson & Jeffers. 1999. Plant Disease 83:1129-1136

***Note*: This medium is less conducive to spore formation by *Phytophthora* spp. than its V8-based counterpart…**

Ingredient	Amount per 1.0 Liter	Amount per 0.5 Liter	A. I. Concentration (PPM)
Basal Medium			
Difco Cornmeal Agar	17 g	8.5 g	
Distilled Water	1000 ml	500 ml	
Amendments			
Delvocid [50% pimaricin]	10 mg = 0.01 g	5 mg	5
Sodium Ampicillin	250 mg = 0.25 g	125 mg	~250
Rifamycin-SV [sodium salt]	10 mg = 0.01 g	5 mg	~10
**Terraclor [75% PCNB]	66.7 mg = 0.0667	33.4 mg	50
**Hymexazol	50 mg = 0.05	25 mg	50
or **Tachigaren [70% Hymexazol]	71.4 mg = 0.071 g	35.7 mg	50

** PCNB and hymexazol are optional and can be omitted [e.g., to make PAR, PARP, & PARH]
- PCNB is particularly useful to inhibit soilborne fungi on soil dilution plates
- Hymexazol inhibits **most** *Pythium* spp. while allowing **most** *Phytophthora* spp. to grow

Directions

1. Add ingredients for basal medium to a 2-L flask; thoroughly mix on a magnetic stirrer with large stir bar in 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
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. Best if plates are used within several weeks—but they will keep for months

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Sources of Amendments Used in Selective Media

These are the sources used by the Jeffers Lab at Clemson University; others sources for these amendments are available…

Amendment	Source	Contact Information
*Delvocid Salt or XT1 (50% pimaricin)	Nelson-Jameson Inc. Marshfield, WI	800.826.8302 www.nelsonjameson.com
Ampicillin (sodium salt)	Shelton Scientific-IBI Peosta, IA (Product No. IB02040)	800.253.4942 www.sheltonscientific-ibi.com
Rifamycin-SV (sodium salt)	Sigma-Aldrich [Product No. R8626]	www.sigmaaldrich.com
Terraclor [75% PCNB]	Chemtura Corp./Crompton Corp	http://www.chemtura.com
**Tachigaren (70% hymexazol)	Gustafson LLC (now part of Bayer Crop Sciences)	www.bayercropscienceus.com/

* Over the years, we have used *Delvocid Instant*—which is a food-grade commercial product that contains 50% pimaricin (also known as natamycin) and was formulated with sugar. It commonly is used in the production of cheeses. This product no longer is available. It has been replaced by *Delvocid Salt* (formulated with NaCl) and *Delvocid XT1* (formulated with xanthum gum). These products are manufactured by DSM Food Specialties (the USA branch of Gist-brocades, a Dutch company), and are commercially available from Nelson-Jameson, Inc—see above. *Delvocid Salt* is only available in 500-gr quantities (~$300.00), and *Delvocid XT1* is available in 100-gr quantities (~$80.00); either one should work well in PARPH-V8.
Please note: Pimaricin also can be purchased through Sigma-Aldrich Co. <*www.sigmaaldrich.com*>.

***Tachigaren* is the formulated fungicide (originally from Japan) that has hymexazol as the active ingredient. We have obtained samples of this fungicide from Gustafson LLC—when they were evaluating the potential of this product for the commercial turfgrass industry. Gustafson decided not to pursue *Tachigaren* as a commercial product so availability of samples may be limited. To complicate matters further, Gustafson now has merged with Bayer Crop Sciences, so any requests for the fungicide would need to go through Bayer…
Please note: Hymexazol also can be purchased through Sigma-Aldrich Co. <*www.sigmaaldrich.com*>.

Plastic Boxes for Storing Petri Dishes in the Incubator

We routinely place petri dishes in plastic crisper boxes in the incubator. Therefore, they do not need to be placed in plastic bags or wrapped with Parafilm. I have been buying these boxes from the same company since graduate school (i.e., for over 25 years!). The company has changed names and locations, but the products have remained the same.

Pioneer Plastics, Inc.
P.O. Box 6,
1584 Hwy 41A, North
Dixon, KY 42409

Phone: 270.639.9133
Fax: (270.639.5882
Packaging Sales: 800.951.1551
web site: *www.pioneerplastics.com*

Here are the products we use:

- 295-C—will hold up to 42 plastic petri dishes: 6 stacks of dishes with 7 dishes per stack
- 395-C—a little sturdier than 295-C; will hold 49 petri dishes: 7 stacks of dishes, 7 dishes/stack
- 398-C—will hold12 petri dishes: 3 stacks of dishes, 4 dishes/stack

Recipes for Media Useful in the Culture and Identification of *Phytophthora* species

V8 Agar & Broth Recipes

V8 agar and broth are excellent growth media for species of *Phytophthora* and *Pythium.*
There are numerous versions of these recipes.
Listed below are the ones we routinely use in my laboratory.
The original "standard" V8A uses 20% buffered, non-clarified V8 Juice--i.e., 200 ml/L.
This often is too rich and promotes excessive vegetative growth.
Most media are more useful if clarified, buffered V8 Juice is used at reduced concentrations.

Clarified, Buffered V8 Juice = clar/buff V8 Juice
To buffer: Add 1.0 g of CaCO3 for each 100 ml of V8 Juice and mix thoroughly on a magnetic stirrer
To clarify/Option 1: Centrifuge at 4000 rpm for 20 min;
then filter through 2 layers of Whatman No. 1 filter paper
To clarify/Option 2: Centrifuge at 7000 rpm for 10 min; then filtration is not necessary
To store: A large volume can be prepared at once and then stored;
freeze desired amounts [50, 100 ml] at -20 C in a Frost-free freezer

Broth vs. Agar
For the recipes below, the only difference between making agar and broth is the addition of agar
For BROTH recipes: Leave out the agar and change the abbreviation accordingly--cV8A to cV8B

Medium	Ingredient	Amount / Liter		Amount / 500 ml	
V8A--20%	V8 Juice	200	ml	100	ml
	CaCO3	2	g	1	g
	Agar	15	g	7.5	g
	Distilled water	800	ml	400	ml
V8A--10%	V8 Juice	100	ml	50	ml
	CaCO3	1	g	0.5	g
	Agar	15	g	7.5	g
	Distilled water	900	ml	450	ml
cV8A--10%	Clar/buff V8 Juice	100	ml	50	ml
	Agar	15	g	7.5	g
	Distilled water	900	ml	450	ml
cV8A--5%	Clar/Buff V8Juice	50	ml	25	ml
	Agar	15	g	7.5	g
	Distilled water	950	ml	475	ml

For all of the above recipes:
> sterilize in the autoclave
> cool medium and dispense into petri plates
> store inverted in plastic bags in a dark refrigerator

Super V8 Agar

References: Chee, et al. 1976. Plant Disease Reporter 60:866-867
Jeffers & Aldwinckle. 1988. Phytopathology 78:328-335.

This medium is ***excellent*** for the production of oospores by species of *Phytophthora* and *Pythium*
>Particularly heterothallic species and others that don't fruit readily

	Chee Recipe = 20% V8				Jeffers Recipe= 10% V8				Modified recipe = 5% V8			
Ingredient	1000 ml		500 ml		1000 ml		500 ml		1000 ml		500 ml	
Clar/Buff V8 Juice*	200	ml	100	ml	100	ml	50	ml	50	ml	25	ml
beta-Sitosterol**	30	mg	15	mg	30	mg	15	mg	30	mg	15	mg
L-Tryptophan	20	mg	10	mg	20	mg	10	mg	20	mg	10	mg
CaCl2-2H20	100	mg	50	mg	100	mg	50	mg	100	mg	50	mg
Thiamine HCl	1	mg	0.5	mg	1	mg	0.5	mg	1	mg	0.5	mg
Agar	15	g	7.5	g	15	g	7.5	g	15	g	7.5	g
Distilled water	800	ml	400	ml	900	ml	450	ml	950	ml	475	ml

*** Clarified, Buffered V8 Juice [=clar/buff V8 Juice]:**
To buffer: Add 1.0 g of CaCO3 for each 100 ml of V8 Juice and mix thoroughly on a magnetic stirrer
To clarify/Option 1: Centrifuge at 4000 rpm for 20 min;
then filter through 2 layers of Whatman No. 1 filter paper
To clarify/Option 2: Centrifuge at 7000 rpm for 10 min; then filtration is not necessary
To store: A large volume can be prepared at once and then stored;
freeze desired amounts [50, 100 ml] at -20 C in a Frost-free freezer

**** beta-Sitosterol:**
Dissolve in 10 ml of EtOH by gently warming over an open flame
Add solution to medium while stirring with a magnetic stirrer
A precipitate will form but this should go back into solution during autoclaving

Comments:
Autoclave [with stir bar in flask] and cool the medium as usual
Continue to stir medium during the pouring process to keep ingredients well mixed
Dispense into petri plates; cool; store plates inverted in plastic bags in a dark refrigerator
For best results--use within 2-3 weeks; although, older plates will remain effective for quite some time

For oospore production:
Incubate plates at 20 C in the dark
Oospores should begin to form in 7-14 days; some "uncooperative" isolates may take 5-6 weeks

S. N. Jeffers ***Clemson University*** ***28 March 2007***