# Phenotype analysiss of developmental stages of *P. infestans*

## A. HYPHAE (radial growth assay)

1. Inoculate 100-mm agar plates with plug of defined size (typically a 4 x 4 mm square or a 4 mm plug made using a cork borer). Place plug near one side of the plate. Normally we use rye-sucrose plates with 1.5% agar, with 4 ml/liter nystatin suspension.
2. Incubate plate at 18C in the dark.
3. Measure radial growth daily, using replicated plates (at least two) and making two measurements per plate (at 45 degree angle).
4. Determine growth rate between day 3 and 5, and divide by 2 to determine the daily rate.

*Measurements should be taken during the rapid, linear phase of growth. Measurements should not be used from the very early or late days: cultures often take a day or two to start growing, and growth slows when the plate is about 3/4 full. All plates used in an assay should be made at the same time (even slight variation in plate wetness or agar concentration can affect growth), and remember to include controls!*

## B. SPORANGIA (sporangia yield assay)

1. Inoculate fresh rye-sucrose plates with your strain. Cultures can either be started from plugs or by spreading sporangia on a plate (typically $10^3$ to $10^4$ per 100 mm plate).

   *If you are comparing different strains, make sure the starting material is of the same age. Typically, a 100 mm plate will yield 10^5 sporangia.*

2. Grow in the dark at 18ºC. If the goal is to count sporangia, normally the plate is grown for 8 days.
3. Harvest sporangia by pouring 15 mls of water on the plate (if you want to make zoospores later, make sure that the water is cold). Use a glass spreader to rub off the spores. Decant the spores into a glass beaker or 50-ml tube. The solution should look milky.

   *If you want to work with the sporangia (not just count them), normally the suspension is poured through a 50 micron nylon mesh, and the collection vessel is kept on ice. If you are analysing many strains, you can use your finger to insert a square of mesh into the top of a 50-ml tube. Note that 15 ml is the suggested volume for a 100-mm plate; use 20-25 if working with a 150 mm plate.*

   *For some experiments, you may want to recover the sporangia in the following solution, which keeps sporangia "happy": Modified Petri's solution: 1X is 0.25 mM $CaCl_2$; 1 mM $MgSO_4$; 1 mM $KH_2PO_4$, 0.8 mM KCl. Normally this is prepared from a 50X stock.*

4. Count spore concentration using a hemocytometer. For some studies, you may also want to record the average size of the sporangia, shape, etc.

## C. SPORANGIA: DIRECT GERMINATION ASSAY

1. Place sporangia in clarified rye broth at 5 x $10^3$/ml. Typically this is done in a 24-well plate, if you are comparing multiple strains.
2. Incubate at 18C for 24-36 hours in the dark.
3. Gently pipette up and down using a wide-bore pipette, place a drop on a hemocytometer, and count.

   *Normally the 24-hr timepoint is sufficient for determining if a strain is altered in direct germination. Since it is hard to evenly pipette germinated sporangia, don't let the culture growth too long before analysis. Measure the percent of sporangia that have germinated. Also keep an eye out for differences in the average germ tube length, the number of "empty" sporangia, or other abnormalities.*

## D. INDIRECT GERMINATION ASSAY (ZOOSPOROGENESIS)

Using sporangia prepared as described in "C", and a pre-chilled plastic tray (see 3 below), induce zoospores as follows:

1. Start with a prechilled suspension of spores (put the spores on ice for 10 minutes).

   *Exposure of sporangia to temperatures above 15C will impair zoospore release. Also, if you are comparing multiple strains, make sure that you treat all identically!!!!*

2. Depending on the number of strains that you will be using, either place the sporangia suspension in a pre-chilled glass petri plate (20 mls/plate) or in a pre-chilled 6-well plate (2 mls/plate).

   *Exposure of sporangia to temperatures above 15C will impair zoospore release. Also, if you are comparing multiple strains, make sure that you treat all identically!!!!*

3. Place the plate(s) in a 10C "incubator". The standard way to make the incubator chamber is as follows: take an autoclave tray, fill it 1/3 of the way with ice, and place a 4-mm plexiglass sheet on top of the ice. Place a small glass thermometer on top of the plexiglass. Cover the autoclave tray with an orange tray; this serves as the "door" of the incubator. After about 15 minutes the thermometer should read 10-11C.

   *Place the plates containing the sporangia ON TOP of the plexiglass, inside the chamber.*

4. After 2.5 hours, count the number of zoospores that were released. Do this as follows: Gently pipette up and down using a wide-bore pipette, place a drop on a hemocytometer, and count (a) the number of ungerminated sporangia, (b) the number of zoospores, (c) the number of empty sporangia.

   *Normally the goal is to compare the rate of zoospore release; normally zoospores start emerging by 90 minutes, so 2.5 is similar to an "endpoint" value. By counting both the number of empty sporangia and zoospores, you can assess both the fraction germination and whether the zoospores are stable (perhaps they might lyse).*

   *Also: keep track of the number of aberrant zoospores, such as those that resulted from incomplete cytoplasmic cleavage. Also check for unusual swimming behaviors, and the fraction that are no longer swimming (which may also include encysted zoospores).*

*In theory, it is possible to measure this phenotype using sporangia washed off a tuft of hyphae, rather than those collected from a plate as described above. In practice, it is very hard to treat strains the same if you do it this way.*

## E. ENCYSTMENT OF ZOOSPORES

1. Pour the zoospores into a plastic tube, filled about half-way. You can do this at room temperature (but treat all samples the same!).
2. If the zoospores are in water, add $CaCl_2$ to 0.5 mM (this is not necessary if using Petri's solution).
3. Vortex at half speed for 60 sec. Wait two minutes and view an aliquot under the microscope. If encystment is poor, vortex another 60 seconds.
4. Gently pipette up and down using a wide-bore pipette, place a drop on a hemocytometer, and measure the number of cysts per ml.

   *Note: the goal here is to both measure the efficiency of encystment and whether zoospores are lysing upon encystment. Compare the concentration of encysted and swimming zoospores to the starting concentration to determine if lysis is occurring.*

## F. CYST GERMINATION AND APPRESSORIUM DEVELOPMENT

1. Encyst zoospores as described above. Place the cysts in 24-well plates. These need to be a brand that is "tissue culture treated."
2. Incubate at 18C in the dark.
3. Using an inverted microscope, measure the concentration of zoospores that formed appressoria.

   *Note: normally the cysts settle on the bottom of the plate, and then form small but noticable germ tubes after 1 hour. By 2 hours, most should have formed appressoria. Sometimes it takes longer, up to 4 hours. Avoid scoring appressoria development after 6 hours since germ tubes may show aberrant swellings that may be hard to distinguish from true appressoria.*

   *Of course, if you are comparing multiple strains, treat them the same and don't forget to include controls!*