TRANSFORMATION OF *PHYTOPHTHORA INFESTANS* BY ELECTROPORATION

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PREPARATION

You should have the following materials ready:

Chilled modified Petri's solution.
Sterile glass petri plates.
Cold 400 ml glass beaker.
50 µM mesh.
5 M LiCl, cold.
Sterile 50 ml screw-cap centrifuge tubes.
Solutions of 33% Percoll and 133% Percoll in modified Petri's solution.
Sterile 10 ml tubes with volume markings
5 ml tubes containing the DNA, chilled.
Chilled electroporation cuvettes (4 mm gap).
Regeneration medium.
Centrifuge reserved and pre-cooled, with swinging bucket rotor.

PREPARATION OF ZOOSPORES FROM *P. INFESTANS.*

1. Inoculate 4-8 fresh 15 cm rye/sucrose plates with 5 agar plugs each of a suitable strain such as 88069. Plan on using 4 plates per experiment, which will provid enough zoospores for 3-5 electroporations.

2. Grow 7-9 days in the dark at 18ºC. *Do not grow longer than 10 days as the zoospore release starts to degrade.*

3. Pour 20 mls of cold Petri's solution (10ºC) on each plate. Use a glass spreader to rub off the spores. Decant the spores into a 300 ml beaker. The solution should look very milky. *Remove an aliquot to count spore yield.*

4. Pour the spore solution through a 50 micron filter, suspended above a 100 mm glass petri plate. You will end up with two plates, each with ca. 40 ml of liquid or 80 ml total.

5. Now you need to induce zoospores. First, put the plates on ice for 30 minutes to quickly cool them. Leave the ice in the 10C room. Then plate the plates on the counter in the 10C room (no ice).

6. After 30 minutes (60 minutes after harvesting the spores), start checking for zoospore release (remove an aliquot to the microscope). You'll have to quickly open the plates in the non-sterile cold room. Then check every 15 minutes. Usually most zoospores are released by 120 minutes after harvesting. Do not let them go longer than 2.5 hours at 10C—you might get more zoospores, but transformation is not as good.

CONCENTRATION OF ZOOSPORES ON PERCOLL GRADIENTS

1. Do the following steps on ice.

2. Take the glass petri plates and put them in a ice bucket, and bring to the hood. Pour the zoospores into the two 50 ml tubes; there should be about 40 mls in each.

3. Add one-fiftieth (1/50) volume of 5M LiCl to each tube of zoospores. Gently invert the zoospores 5-6 times to mix in the LiCl. Note the time to the nearest 15 seconds. Also, remove an aliquot to count, but do this later—see step 7.

4. Immediately use the electric pipettor to gently underlay 5 ml of 33% Percoll in modified Petri's beneath each aliquot of zoospores. *Go slowly to avoid mixing the layers—it is important to not have too much LiCl in the lower layer, since this will interfere with electroporation.*

5. Next, underlay 5 ml of 133% Percoll in modified Petri's.

6. Place the tubes in the precooled centrifuge rotor. You'll need to run the rotor without its lid.

7. EXACTLY 5 min after you mixed in the LiCl, turn the centrifuge on. Set for 1800 rpm for 5 min at 4ºC. Use maximum acceleration and maximum brake. While waiting, you have time to count the zoospores to determine the "prespin" yield.

8. Immediately the centrifuge stops, gently remove the tubes, place on ice, and return them to the hood. You should be able to see a pale brown layer at the interface between the 33% Percoll and the 133%. Using a dark background may help in seeing the bands. If you can't see ANY bands you do not have enough zoospores to continue.

9. Using the electric pipette (5 ml pipette) or a P-5000 pipettor, gently remove most of the 133% percoll layer below the zoospores. Stop as soon as you see zoospores entering the pipette tip. This step greatly improves theability to collect the zoospores in a concentrated band. Discard this layer.

10. Re-insert the 5 ml pipette to the tube, and slowly remove the zoospore band. This should be about 2.5 ml per tube---minimize the volume!! Some liquid from the upper LiCl layer may enter the pipette due to hydrostatic pressure; gently expel this before removing the zoospores. Stop before you see non-zoospore containing liquid entering the tip. Pipette the zoospores into a sterile 10 ml tube with volume markings. Remember to leave on ice.

11. Repeat steps 9 and 10 for each centrifuge tube, pooling the zoospores.

ELECTROPORATION

1. Each electroporation will use 600-800 µl of zoospores (800 is preferred) with 15-40 µg of DNA. Divide the zoospores into separate prechilled tubes containing the DNA. There should be 0.5 to 2 x 10^7 zoospores per tube (don't determine this yet—but save some zoospores in the original tube to (i) count after the electroporation, and (ii) for a no-electroporation control).

2. Invert the tubes twice, then pipette ca. 800 µl into each cuvette. Cap the cuvette.

3. Wipe the cuvette dry with a tissue, and electroporate: 550 V, 50 µF. *This may not be optimized; 500-600 is optimal.* Record the time constant. It should be between 6 and 10 ms. If it is <6, there is too much LiCl in the zoospore solution.

4. Immediately remove the cuvette from the electroporator and place on ice.

5. When done electroporating all cuvettes (should take <1 min total), add 800 µl of

regeneration media. Then pipette out the zoospore mixture into a 15 ml tube (on ice), using a 1 ml pipette. Add more regeneration media, to a total of 10 ml. Do the same for a no-electroporation control. *(I usually use 1/4 the volume of zoospores and media for this control).*

6. Also add 25 µg/ml vancomycin, 50 µg/ml ampicillin, and 4 µl/ml of nystatin.

7. At this point, you have time to count the number of zoospores recovered from the Percoll gradient.

8. When you are all done with the experiment, reuse the cuvettes: rinse with water, fill with ethanol overnight, then dump out the ethanol and let dry in the hood.

REGENERATION AND PLATING.

1. Invert the 15 ml tubes gently and place on their side at 18C for 16-20 hours. *Don't go longer, as the hyphae will mat together.*

2. After 20 hours, record the concentration of germinated zoospore cysts. Multiply by the volume, and divide by the original number of zoospores to determine regeneration. It should be 80-90% for the nonelectroporated control, and 30-60% for the electroporated samples.

3. Plate the regenerated zoospores on selective medium. Spin the zoospores at 1500 rpm at room temperature, 5 min, using the bench-top centrifuge. Decant all but 1.8 ml of media. GENTLY resuspend.

4. Spread ca. 0.3 ml on each of 6 plates: rye-sucrose agar plates containing 7 µg/ml G418, 6, 25 µg/ml vancomycin, 50 µg/ml ampicillin, and 4 µl/ml of nystatin.

5. Incubate at 18C. Leave right-side up for ~1 day until the liquid is absorbed. Then invert. Colonies will appear after 8-12 days.

SOLUTIONS

Modified Petri's solution. Make a 50X stock. 1X is 0.25 mM $CaCl_2$; 1 mM $MgSO_4$; 1 mM KH_2PO_4, 0.8 mM KCl [Note that regular Petri's has 2.4 mM $Ca(NO_3)_2$ instead of 0.25 mM $CaCl_2$]

33% Percoll in mod. Petri's. (3.3 ml 100% Percoll; 200 µl 50X Petri's-Ca; 25 µl 100 mM $CaCl_2$, 6.6 ml water---this is enough for two gradients, but you can make more)

133% Percoll in mod. Petri's. (8.9 ml 150% Percoll; 200 µl 50X Petri's-Ca; 25 µl 100 mM $CaCl_2$, 1 ml water—this is enough for two gradients, but you can make more. Also, this can be made from something besides 150% Percoll, if you do the calculations correctly)

Note: Percoll can be autoclaved before salts are added, but try to avoid it.

5 M LiCl

Regeneration medium: cleared liquid rye sucrose plus 100 mM mannitol, 1 mM KCl, 2.5 mM $CaCl_2$