

LABORATORY MANUAL FOR *P. INFESTANS* WORK AT CIP-QUITO

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Many of the methods presented here were developed in other laboratories, in particular Dr. Fry's laboratory in Cornell, U.S.A., and Dr. Shaw's laboratory in Bangor, G.B. Many thanks to these and other colleagues who have offered useful advice for our work on *Phytophthora infestans*.

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1. ISOLATION OF *P. infestans* FROM INFECTED TISSUE

There are different ways of isolating *P. infestans* from infected tissue but two common ways are 1) to transfer fungal hyphae and sporangia directly onto medium in a petri dish, and 2) to place infected plant tissue on selective medium. The first of these generally involves growing the fungus on potato tubers and the second on tuber or leaf tissue. In our experience, potato genotypes are easier to isolate with method 1, and tomato isolates with method 2. In any case, it is always easier to isolate from a recent infection which is just beginning to sporulate.

P. infestans is relatively easy to keep alive by repeated inoculations on living tissue. On the other hand, *P. infestans* is sometimes difficult to get into pure culture. Therefore, keep your isolate alive on living tissue until it is successfully purified.

1.1 Isolation *P. infestans* from infected potato leaves

1. Sporulating lesions on leaf tissue taken from the field are washed in fresh water and placed in a humid chamber (inverted petri dish with water agar) with the leaf's abaxial side up.
2. Plates are incubated at 15-18°C for 1 day or until fresh sporulation appears.
3. Small pieces of infected tissue from the sporulating border of the lesion are cut and placed under potato slices in an empty petri dish.
4. Dishes are incubated at 15-18°C for 1 week, until there is abundant sporulation on the upper side of the slice.
5. To re-inoculate leaves, pick sporangia from the top of the tuber and place them in a drop of water on a potato leaf or another tuber slice.

You can repeat steps 2-5 several times to keep your isolate alive (Figure 1).

1.2 Isolation of *P. infestans* from infected tomato leaves

1. Sporulating lesions on leaf tissue from the field are washed in fresh water and placed in a humid chamber with the leaf's abaxial side up.
2. Plates are incubated at 15-18°C with a 14hour light period for 1 day or until sporulation appears.
3. Small pieces of infected tissue from the sporulating border of the lesion are cut out and placed on top of a drop of water on the abaxial side of tomato leaves in a humid chamber (upturned Petri dish containing water agar).

4. Dishes are incubated at 15-18°C with a 14 hour light period for 1 week, or until there is abundant sporulation.
5. To re-inoculate leaves, pick sporangia from the top of the leaf and place them on a drop of water on a tomato leaf, or place small drop of water directly onto the sporulating lesion, wash the lesion several times with the same drop of water, then inoculate the abaxial surface of a new leaflet with the sporangial suspension obtained.
6. You can repeat steps 2-5 several times to keep your isolate alive.
This can be done as in Figure 1, but by substituting a tomato leaf for the tuber slice.

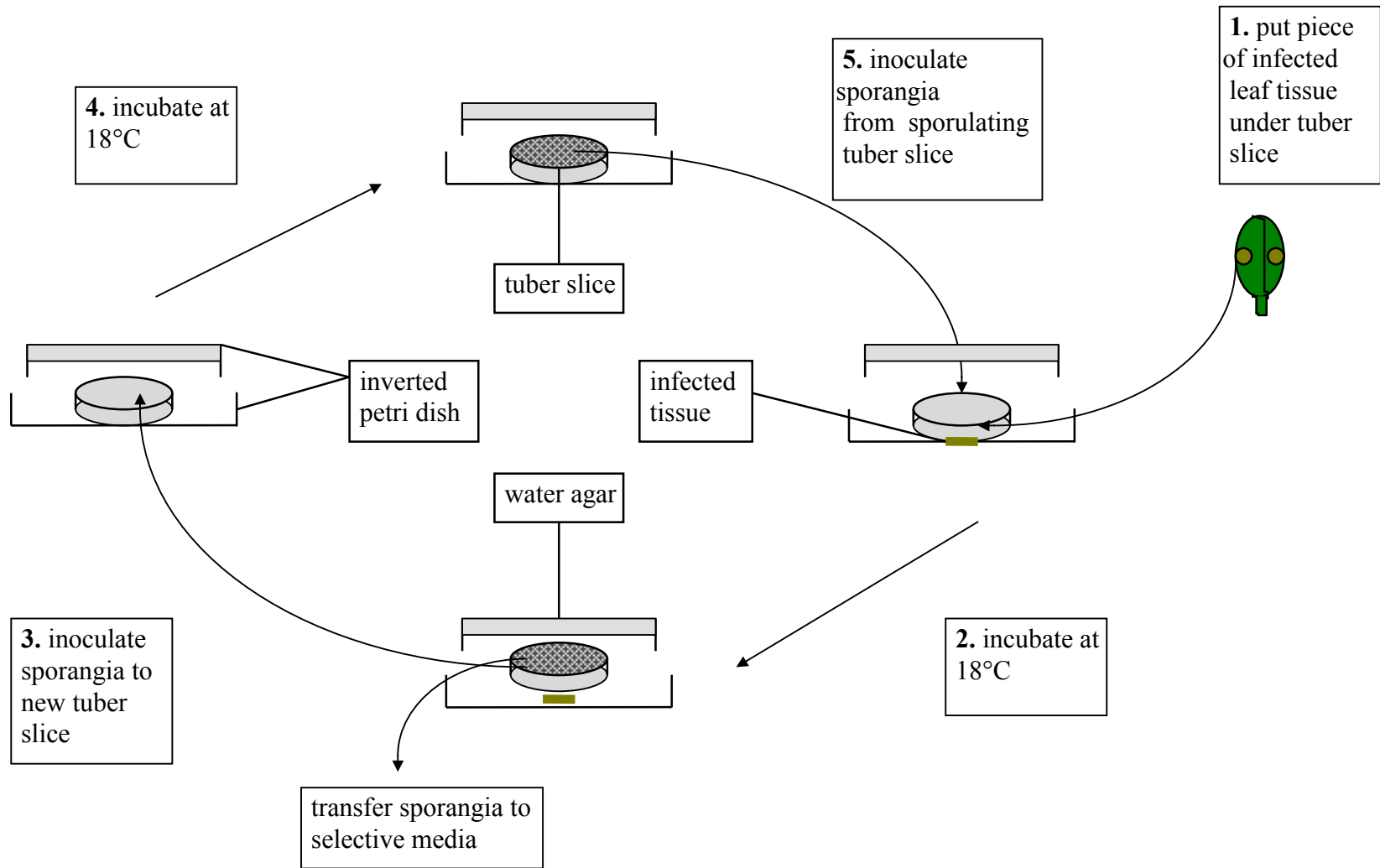


Figure 1.

1.3 Isolation of *P. infestans* from infected tubers

If isolating from infected tubers, slice the tuber open where infection has occurred and place in a moist chamber until sporulation occurs; then follow steps on section 1.4.

1.4 Isolation in agar

After transfer from potato slices. When clean inoculum appears on the upper side of an infected tuber slice or leaf, the sporangia are harvested in a flow chamber, by picking them up with an inoculating needle and placing the sporangia on selective medium. Do not touch the potato slice or leaf with the needle. The use of a stereoscope is helpful.

Directly from infected leaf or stem tissue. It is possible to isolate the fungus directly from the infected leaf tissue, but it is advisable to produce fresh inoculum at least once. To isolate directly, a small piece of infected leaf from the sporulating border, including a little bit of green tissue, is cut out and passed through a 5% commercial bleach solution for 30 seconds, then rinsed in sterile distilled water twice, and dried off with sterile filter papers. The leaf pieces are then placed on top or inside a selective medium. The plates are incubated 18°C for 5-10 days, or until the fungus starts growing and feeding on the agar. Hyphal tips are then transferred to V8 or Rye B agar plates.

2. MULTIPLICATION OF *P. infestans*

We have two methods for multiplying *P. infestans*: 1) on potato slices or leaves, and 2) on pure culture on agar plates.

2.1 On Potato Slices or Leaves

We frequently multiply *P. infestans* on potato slices or leaves; primarily for inoculum production, but also for maintenance.

2.1.1 Harvesting of sporangia and zoospores

1. Sporangia are washed from the upper side of a sporulating lesion on a potato slice or tomato leaf with distilled water, and passed through a 30 micron mesh filter to remove mycelium and other debris.
2. The filtrate is then passed through a 10 micron mesh filter, which traps the sporangia. These are washed several times with clean water, and then collected from the filter with a small amount of distilled water.
3. This sporangial suspension is incubated at 6°C for 2 hours to promote zoospore release.

If one wants to separate sporangia from zoospores, the suspension is again passed through the 10 micron mesh filter once the zoospores have been released, and the filtrate, containing only zoospores, is collected.

The zoospore suspension is inoculated onto tuber slices or potato leaves (in the case of inoculum from potatoes) or tomato leaves (in the case of tomato inoculum) to keep the fungus growing.

2.1.2 Inoculation of tubers

1. Select tubers from susceptible varieties, medium sized if available, without rots, severe damage or green coloration.
2. Wash the tubers thoroughly and let them dry.
3. Surface sterilize the tubers by dipping the them in 70% alcohol for a few seconds, and burning off.

4. With a knife dipped in alcohol and burned off, cut the potato into about 1cm thick tuber slices. Each slice should have two cut surfaces. The outer most parts of the tubers are discarded.

Place the potato slices on sterilized wire netting in shallow plastic boxes with damp filter paper at the bottom. The netting should be at least 1cm above the bottom of the tray. Two drops of sporangia or zoospore suspension are placed on the tuber slices, the boxes are closed in order to create a moist chamber, and incubation follows at 15-18°C for 1 week.

2.1.3 Inoculation of leaves

1. Cut leaflets of fully expanded potato or tomato leaves, from greenhouse grown plants which are not flowering, and place them in fresh water. Leaves must be healthy with no signs of disease or stress.
2. Rinse the leaves and pat them dry with paper towel.
3. Place leaves abaxial side up in the lids of inverted petri dishes containing water agar, and inoculate with two drops of sporangial or zoospore suspension of *P.infestans*.
4. Incubate at 15-18°C for 1 day in the dark, then for 6 days with a 14 hour light cycle.

For maintenance of cultures of *P. infestans* either one of these two procedures may be repeated once a week. Only spores formed on the non-inoculated side of the potato slices or leaves are harvested. For inoculum production, spores from the inoculated side of the slices or leaves may also be harvested if no bacterial colonies are visible.

A note on contamination! Be careful with contamination of tuber slices (and to some extent leaves) with Erwinia and other organisms that may attack potato. Always inoculate tuber slices with very clean (filtration as above) and very dilute amounts of inoculum. A 10 micron drop with about 50 zoospores is plenty of inoculum for tuber slices and will guard against contamination. Also, you may just transfer pieces of hyphae and sporangia as shown in Figure 1.

2.2 On agar plates

Basically all these recipes come from Catin and Jinks [Caten, 1968 #3268; Caten, 1970 #2098], and may have been slightly modified by Bill Fry's laboratory in Cornell.

2.2.1 V8-based media

Ingredients (for 1 liter)	10% Clarified	10% Unclarified	15% Unclarified	20% Unclarified
V8 juice	150ml	100ml	150ml	200ml
CaCO ₃	1.5g	1g	1.5g	2g
β-sitosterol	0.05g	0.05g	0.05g	0.05g
Agar	15g	15g	15g	15g
Purpose	Mating type	Sporulation, Selective*	Sporulation	Sporulation

1. Combine V8 juice and enough distilled water up to bring up to 1 liter.
2. Add CaCO₃ and β-sitosterol and mix well.
3. Then add agar and autoclave at 15 psi for 20 minutes.

Stir medium while dispensing to insure good mixing of CaCO₃.

For the clarified medium, first centrifuge the 150ml of V8 for 5 minutes at maximum speed, and then use 100ml of supernatant and follow the recipe above.

* The selective media is prepared adding antibiotics (see below).

2.2.2 Rye based media

Ingredients (for 1L)	Rye A	Rye B
Rye	60g	60g
Sucrose	20g	20g
β-sitosterol	0	0.05g
Agar	15g	15g
Purpose	Maintenance	Sporulation, Selective*

* The selective media is prepared by adding antibiotics (see below).

a) Rye A

1. Soak rye grains in approx. 100ml distilled water for 36 hours. If less dH₂O is used, grains seem to germinate more quickly (24-30 hours).
2. Pour off and reserve liquid.
3. Blend the swollen grains for around 2 minutes (can add some dH₂O), and incubate for 3 hours at 50°C in distilled water. Don't modify time.
4. Filter through four thickness of gauze and discard the sediment.
5. Combine the original supernatant and the filtrate with agar and sucrose. Adjust volume to one liter.
6. Autoclave at 15 psi for 15 min., pour plates.

b) Rye A slants

1. Follow steps 1-5 above.
2. Heat agar on hot plate until agar begins to melt., and put 2-3ml media/tube.
3. Cap tubes and autoclave at 15 psi for 15 min.
4. Slant the tubes and do not move until they have cooled.

c) For Rye B

1. Soak grains in distilled water for 36 hours.
2. Pour off and reserve liquid.
3. Boil the rye grains for 1 hour in enough distilled water to cover the grains.
4. Strain through 4 thickness of gauze, and combine filtrates.
5. Add sucrose, agar and β -sitosterol, then make up to 1 liter.
6. Autoclave at 15 psi for 15 minutes.

Note: Check water level often while boiling the rye, it boils off very fast! If all the water does boil off do not pour more water into dried out and very hot beaker (probably at this point containing burnt rye), it will crack.

2.2.3 Clean up/Selective media

We have two different selective media, one is used in Cornell, and the other developed by Hans Hohl [Hohl, 1991 #3726].

a) For the Cornell medium:

Antibiotics	
Vancomycin	100mg/L
Polymixin B	50mg/L
Ampicillin	200mg/L
Rifampicin	20mg/L
PCNB (75% WP)	67mg/L
Benlate (50% WP)	100mg/L

1. Prepare 10% unclarified V8, and after autoclaving let the media cool.
2. Add the antibiotic mix above, stir, and pour plates.

b) For the Hohl medium:

Antibiotics	
Griseofulvin	20mg/L
Nystatin	19mg/L
Benlate (50%WP)	10mg/L
Methoxypurine	5mg/L
Rifamycin	30mg/L
Nalidixic acid	5mg/L
8-azaguanine	40mg/L
Neomycin	30mg/L

1. Prepare Rye B agar and after autoclaving let media cool.
2. Mix the antibiotics in DMSO, and add them to the medium, mix thoroughly, and pour plates.

You can also prepare antibiotic stocks in 10ml of DMSO and store 1ml aliquots in the freezer. Use 1ml of mix per 1 liter of media.

2.2.4 Other media

a) Pea Broth

Ingredients	
Frozen or fresh peas	120g
Purpose	Harvest mycelium

1. Autoclave peas in approximately 1 liter of distilled water for 15 min.
2. Strain peas from broth using 4 layers of gauze.
3. Bring volume of broth up to 1 liter and autoclave for 15 min.
4. Pour plates.

b) Unclarified V8/Lima bean Agar

Ingredients (for 2 liters)	
Baby lima beans	160g
V8 juice	200ml
CaCO ₃	2.8g
Agar	30g
Purpose	Sporulation

1. Autoclave baby lima beans in 400ml of distilled water for 10 min.
2. Strain through gauze and discard the lima beans.
3. Add CaCO₃ to V8 juice bring the volume of the combined juices to 2 liters with distilled water.
4. Adjust pH to 6.0 with KOH or HCl.
5. Add agar and autoclave at 15 psi for 15 minutes and pour plates.

3. STORAGE OF *P. infestans*

3.1 Liquid Nitrogen

We store a dense suspension of sporangia freshly harvested from tubers or leaves.

1. The sporangia are washed with distilled water and concentrated in a filter (10 micron mesh).
2. The suspension is then mixed with 15% DMSO in the filter after the last wash. Wear gloves and work in a well ventilated area.
3. Once the suspension is in the vials they are cooled slowly in an alcohol bath with controlled cooling and constant stirring for 3-4 hours until they reach -50°C . The controlled cooling is done by an immersion cooler. We use a Neslab (cc 60 IIA) cooler and a Neslab Agitainer for holding and stirring the alcohol. Both are available via the big lab equipment vendors.
4. Quickly transfer the vials into the liquid nitrogen.

Thawing involves dropping the vials in tap water (ca. 20°C) for a few minutes and then the suspension can be put directly on potato slices.

Alternate method from Cornell

1. Put 1 ml 15%DMSO in 2 ml cryovial, autoclave for 15 min.
2. Add 5 plugs (cut with sterile instrument) taken at random 1cm from margin of 1-2 week old plate. A 40mm diameter colony will make 10 plugs if entire colony is used.
3. Snap vials onto cane, when canes are filled, put onto cardboard sleeve and into refrigerator in an open pipette can (4-5 canes/can); after the last cane is in the can, let them all sit for an additional 10 min.
4. Put top on pipette can and place into -80°C for 60 min, then place canes slowly into liquid nitrogen canister.

You can also put the cryotubes in a box and then in a Styrofoam container. Place this in the refrigerator for 30 min. then in the freezer for 25 min., afterwards in -80°C for one night. They can then be put in liquid nitrogen.

To thaw, put in tap water (same as CIP) and then the plugs are transferred to fresh medium.

For both the Cornell and the CIP procedure there is now in the market a container from Nalgene (Mr. Frosty) in which the vials are put in an isopropyl alcohol bath, put in an 'ultrafreezer' (-80°C) for 4 hours and then the vials are put onto canes and in the liquid Nitrogen. The method claims it's rate of cooling is 1°C/min. The vials can either have agar plugs or spore suspension. This information may be useful [Cunningham, 1973 #4843; Long, 1978. #4844].

3.2 Agar slants

Isolates are maintained in Rye A agar slants (see media recipes) stored at 15°C. A small actively growing plug of mycelium is put at the bottom of the slant and tubes are sealed with parafilm. New transfers are made every 4-6 months.

4. METALAXYL TESTS

Use 10% unclarified V8 media with the corresponding metalaxyl concentration. The fungicide is prepared in a stock of 100mg/ml. It is made by dissolving 1.1g of 90.6% technical grade metalaxyl in 10 ml of DMSO. Allow medium to cool to about 50°C before adding the metalaxyl.

Three different metalaxyl concentrations are tested:

Concentration	0µg/ml	5µg/ml	100µg/ml
10% V8	1000 ml	1000 ml	1000 ml
DMSO	1.0 ml	0.95 ml	0 ml
Metalaxyl stock	0 ml	0.05 ml	1.0 ml

Set up duplicate plates of each of the three concentrations of metalaxyl.

1. Cut uniform size agar plugs from actively growing *P. infestans* cultures and put one plug per plate of metalaxyl. We use the end of sterile large volume Pasteur pipets for a 9 mm size plug.
2. After seven days measure the growth of the fungal colony at right angles, two diameters through the center of each plate. We usually include two known isolates in our tests as checks: one resistant and one sensitive.

Metalaxyl resistance is determined as follows:

Resistant: both 5 and 100µg/ml \geq 40 % growth of 0µg/ml;
Intermediate: 5µg/ml \geq 40 % growth of 0µg/ml
Sensitive: both 5 and 100µg/ml $<$ 40 % growth of 0µg/ml.

5. DETACHED LEAF INOCULATION FOR VIRULENCE TESTS

Virulence is the ability of the pathogen to infect and reproduce on a plant with an identified gene for vertical resistance. This is usually tested with an inoculation on detached leaves. There are several ways of doing this but at this writing ours most resembles that of Cornell. The CIP approach is given below and then the Cornell procedure following.

5.1 CIP procedure for testing virulence

1. Cut leaflets of differentials in the morning. Leaflets should be taken from the upper third of 6-8 weeks old plants (before flowering) and completely healthy. Put the leaflets in plastic bags with water to transport to the lab.
2. Use 2 leaflets per differential per isolate and place them abaxial side up in petri plates with water agar on the top. It is better to do no more than 10 isolates at a time.
3. A sporangia suspension is prepared by washing one week old inoculated tuber slices or leaves.
4. Incubate suspension at 5°C to promote zoospore release, and once the zoospores are swimming pass the spore suspension through the 10 micron mesh filter, and collect filtrate containing only zoospores.
5. Calibrate zoospore concentration to 2000 per ml with a hemacytometer. Count two sets of five grids for a total of 20 zoospores. $10 \text{ grids} \times 1000 = \text{zoospores} / \text{ml}$.
6. Place one 10µl drop of the calibrated inoculum on each side of the midrib of the leaflet.
7. Incubate the petri plates at 15-18°C in the dark, then with a 14 hour light period starting the second day after the inoculation, for 6 days.
8. Assess virulence on the sixth day by determining a compatible or incompatible interaction.

5.2 Cornell procedure for testing virulence

The condition of the potato differentials is very important. Plants should be grown only in the green house and only from October to early April. During these months the temperature and the photo period may be controlled (cooler temperatures and a 16 hour photo period are required to maintain plants for virulence testing). If the plants become infested with whitefly Temik may be used with negligible affect on testing.

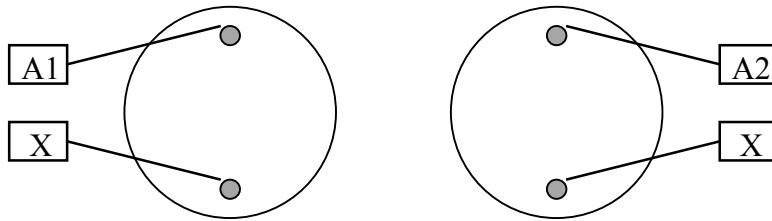
1. Inoculate plates of 10% V8. (If the isolate grows poorly on V8, rye B with β -sitosterol or Pea agar with β -sitosterol maybe used instead).
2. Between 1 1/2 and 3 weeks plates become ready for the virulence assay. (Using the binocular microscope, one must observe the plates in question to determine if they have produced enough sporangia for the assay. Plates may be used as soon as they have produced adequate numbers of sporangia. It is a good idea to check and mark the plates to be used about 2 days before you are planning to perform an assay. Plates older than three weeks should not be used. Unless you are only testing for a couple of virulence phenotypes, you will want to limit the number of isolates being tested to less than 10 at any particular time.).
3. Select leaves the morning of the assay. (Leaves selected for the assay should be dark green turgid leaves showing no signs of any disease. Leaves should never be taken from tuberizing or senescing plants. Selected leaves should be placed in bags with water in a Styrofoam box for transportation back to the lab. This protects the plants from the cold and maintains turgor.).
4. Use 4 leaflets per virulence phenotype. Place 2 leaflets into the lids of petri dishes, randomizing leaflets so that plates do not contain two leaflets from the same leaf. Leaflets may be placed upside down in order to hold the drop of spore suspension better. (Remove any condensation that may have collected on the petri plate lids beforehand.).
5. Harvest sporangia using 2-5 ml of sterile water. (The sporangia should detach from the mycelium on contact with water).
6. Determine spore concentration with a hemacytometer. Count two sets of five grids (10 grids x 1000 = sporangia / ml). Use 40,000 - 100,000 sporangia per ml for the virulence assay. (Spore concentration should be determined right after harvest because within an hour sporangia may start to germinate into zoospores complicating this task. After the spore concentration is determined, the spore suspension may be placed in 4°C until ready to be used (up to 3 hours)).
7. Inoculate each leaflet with one drop of the spore suspension using a Pasteur pipette. (Be careful that you don't knock the drop off of the leaflets while handling.).
8. Place plates in the 18°C chamber with a twelve hour photo period, 24 hours after inoculations, wrap the plates in parafilm.
9. Six days after inoculation, assess leaflets for virulence.

Note: Each isolate should be tested at least twice. If two plates give enough sporangia for the first test the other two plates may be used to repeat the test.

Leaves should be incubated in large petri plates with water agar (plates should be no smaller than 140mm x 20mm).

6. MATING TYPE TEST

Use 10% clarified V8 medium. Place an agar plug of an actively growing isolate you want to test on one side of a petri dish, and a plug of a known A1 isolate at the other side. In another petri dish do the same with a known A2 isolate. Seal the dishes and incubate in the dark for 14-21 days, until the 2 colonies have come in contact with each other, and look for oospores using the microscope.



7. ISOZYME ANALYSIS

We are using 3 methods for this analysis: 1) potato starch gels, and 2) cellulose acetate gels, and 3. polyacrylamide gels. We work with two enzymes: Glucose-phosphate dehydrogenase and Peptidase. The starch and cellulose acetate methods are basically Cornell's procedures.

7.1 Starch gel electrophoresis

7.1.1 Grinding and protein extraction

1. Inoculate pea broth plates (2 plates/isolate; 2-3 plugs/plate) about 10 days to 2 weeks ahead.
2. Harvest mycelium (putting all plates of the same isolate together) and dry by vacuum filtration. Place into a 1.5ml Eppendorf tube, appropriately labelled, and put each tube on ice.
3. Add TC 7 gel "grinding buffer" to each tube. The amount of buffer may vary according to the amount of mycelium, from 100 to 200 μ l.
4. Grind with a hand drill which fit snugly inside the eppendorf tube.
5. Centrifuge tubes at high speed 1 minute. They are ready to be used directly or kept in freezer.

Lyophilized mycelium can also be used. You would need only a small amount, just the tip of a spatula, about 1mg, add less grinding buffer, shake well and centrifuge; the samples are now ready to use.

Buffer	Chemical	Molarity	g/L	g/4L
TC 7	Trisma base	0.135	16.4	65.6
(grinding buffer)	Citric acid	0.04	9.0	36.0
(adjust pH to 7 with KOH or HCl)				

Measure 70 ml of solution and take to 1000ml.

7.1.2 Making the Gels

1. Make up buffers the night before (gel and electrode) and store them in refrigerator. Keep all buffers refrigerated.

Buffer	Chemical	Molarity	g/L	g/4L
TC 6 (GPI) (electrode)	Trisma base	0.135	16.4	65.6
	Citric acid	0.04	9.0	36.0
(adjust pH to 6 with KOH or HCl)				

Buffer	Chemical	Molarity	g/L	g/4L
TC 8.0 (PEP) (electrode)	Trisma base	0.687	83.2	332.8
	Citric acid	0.157	33	132
(adjust pH to 8.0 with KOH or HCl)				

Buffer	Chemical	Molarity	g/L	g/4L
HIS 6 (GPI) (gel)	Histidine-HCl	0.01	2.1	8.4
(adjust pH to 6 with KOH or HCl)				

Buffer	Chemical	Molarity	g/L	g/4L
TC 8.0 (PEP) (gel)	Trisma base	0.023	2.79	7.27
	Citric acid	0.005	1.05	3.15
(adjust pH to 8.0 with KOH or HCl)				

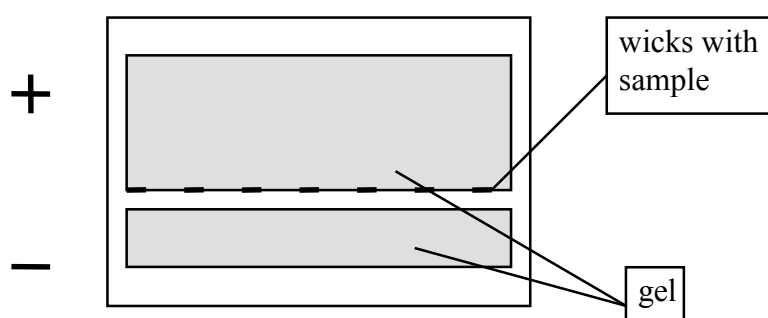
2. Weigh 24g of sifted potato starch and put in 500ml vacuum flask.
3. Clamp plastic bars around edges of the glass plates, make sure edges of the bars
4. touch each other at the corners, and put on top of plastic trays on a level table

Make gels:

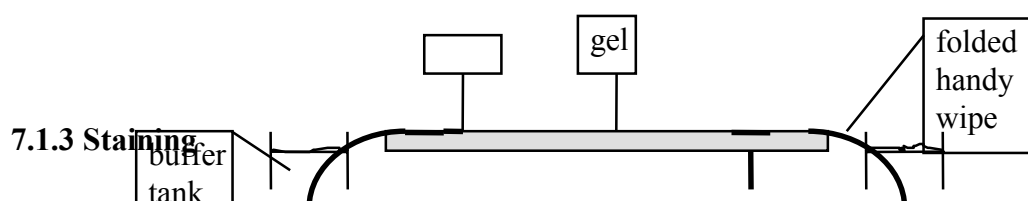
- a) add 70ml of the gel buffer to the starch, and stir
- b) begin to heat 130ml of buffer in a 500ml volumetric flask
- c) when buffer is boiling vigorously, pour into starch (swirling the starch mixture constantly)
- d) replace the mix onto the hot plate and allow to cook, with occasional swirling, until it is clear and thinner and bubbling actively
- e) remove bubbles by attaching flask to vacuum pump

- f) pour gel into plate with a circular motion and remove any bubbles or lumps with a Pasteur pipette
5. Cut wicks of thick blotting paper.
 6. When gels have set, cover gel with plastic wrap.
 7. Get samples and marker dye ready, put them in an ice water bath.
 8. When gels have completely set, remove the two long (lengthwise) bars from tray.
 9. Slice the gel lengthwise about 5cm from edge.
 10. Pull slice back about 1cm, and insert wicks soaked in appropriate sample onto gel. Leave room at the end of the gel for the marker dye (add after other wicks).
 11. When wicks are on, firmly push slice back up against the wicks leaving no air bubbles.
 12. Fill buffer tanks with appropriate electrode buffer (about 1/2 full) and put in handi-wipes folded twice with folded side up.
 13. Put handi-wipes onto both sides of the gel, making sure that they are straight and covering about 2 cm of the gel. Cover the gel with plastic film to prevent drying. Attach electrodes. Fill the tanks with more buffer. The level of buffer should be up to the electrode clip, but NOT touching. Turn on power and allow to run for about 15 minutes or until dye has travelled about 1 cm through gel.
 14. Then turn off power. Remove wicks. Push the two parts of the gell firmly bak together. Turn on power again and allow to run for about 14 hours. For GPI set amperage at 75; for PEP set amperage at 30.

top view



side view



Prepare the following staining buffers and keep them refrigerated.

Buffer	Chemical		g/L
Tris-MgCl (GPI) (staining)	Trisma base	0.1M	12.1
	MgCl ₂	0.1%	1.0

Buffer	Chemical		g/L
TBE 8.7 (PEP) (staining)	Trisma base		21.8
	Boric acid		6.2
	Na EDTA		1.15

Measure 250ml of solution and take to 1000ml with distilled water

An agar overlay staining method is used. Mix all reagents in corresponding buffer and add dissolved agar last. Pour staining solution over gel, let set, incubate at 37°C preferably in the dark for a few minutes, and score.

Glucosephosphate isomerase (GPI)	Amount
Tris-MgCl pH 7	10ml
Fructose-6-phosphate	50mg
NAD	20mg
MTT*	10mg
PMS*	5mg
Glucose-6-Phosphate	50 units
Dehydrogenase	
Agar 1%	10ml

* Stocks can be made of NAD, MTT and PMS:

7.1.7. NAD 200mg 10ml H₂O take 1ml

MTT → 100mg + 10ml H₂O → take 1ml

PMS → 50mg + 10ml H₂O → take 0.2ml

Peptidase (PEP)	Amount
TBE 8.7 gel	10 ml
Glycyl leucine	50mg
O-dianisidine	15mg
Peroxidase	4000 units
a-amino acid oxidase	1.5 units
Agar 1%	10ml

7.2 Cellulose acetate electrophoresis

7.2.1. Preparation of Tissue Samples

Tissue can be from mycelia from broth cultures, scraped off plates or slants, or from washed sporangia from infected leaves or tubers. A very small amount is needed.

1. Put sample in 1.5 ml tube with 100 μ l of distilled water, grind the tissue with a plastic or teflon pestel adapted to a hand drill.
2. Centrifuge for 1 minute to pellet cell debris. The extracts must be chilled before use to avoid enzyme degradation, or frozen for later use .

7.2.2. Gel and buffer preparation

Buffer	Chemical	Molarity	g/L
TG x1	Trizma base	0.025	3g
pH8.5	Glycine, free base	0.192	14.4g

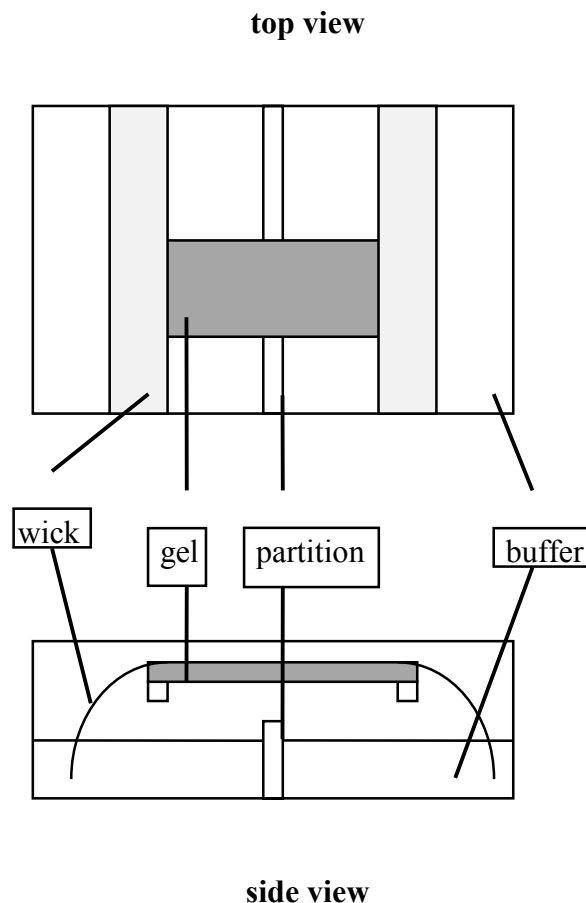
Place several cellulose acetate gels on rack and slowly fill the reservoir with electrode buffer. Care must be taken to avoid air bubbles and splashes. The gels must soak at least 20 minutes before use and can be kept refrigerated for several days.

7.2.3 Gel running

All equipment must be kept spotlessly clean.

1. Fill electrophoresis chambers with electrode buffer and soak in paper wicks. Position the wicks over the supporting rails.

2. Place 8 microliters of sample on wells of sample plate.
3. Take one gel from buffer and blot dry between two blotting papers to remove excess buffer. Place gel on aligning base over a small drop of water to help the gel stay in place. Work quickly, or the membrane will dry out.
4. Pick up samples with applicator 2 - 4 times, and gently place the applicator in the guides and depress for a few seconds to transfer samples to the cellulose acetate gel. Place a small amount of marker dye (bromophenol blue) next to one extreme of samples.
5. Place gel over the moistened wicks cellulose side down with origin at cathode rail. Ensure a good contact between gel and wicks by placing 2 glass microscope slides on top of the gel.
6. Run the electrophoresis at 200 volts (and about 2mA) for 20 minutes for GPI and 15 minutes for PEP.



7.2.4 Staining

We use the overlay method. It saves staining reagents and produces less amount of toxic waste.

To stain, mix ingredients in buffer and add agar last. Pour solution over gel to cover, incubate until bands appear, then rinse with cold water. Score gel and then dry quickly with hair dryer to save as reference.

Quantities for 2 gels:

Glucose-6-phosphate isomerase (GPI)	Concentration	Amount
Tris-HCl, pH 8.0	0.05M	1.5 ml
Fructose-6-phosphate	20mg/ml	5 drops
NAD	3mg/ml	1 ml
MTT	10mg/ml	5 drops
PMS	2mg/ml	5 drops
Glucose-6-Phosphate Dehydrogenase	1U/ μ l	2 μ l
Agar, @ 60°C	1.4%	2 ml

Peptidase (PEP)	Concentration	Amount
Tris-HCl, pH8	0.05 M	2 ml
Glycyl-leucine	15mg/ml	10 drops
Peroxidase	1000 U/ml	5 drops
o-Dianisidine	4mg/ml	8 drops
MnCl ₂	20mg/ml	2 drops
a-amino acid oxidase	10 U/ml	5 drops
Agar, @ 60°C	1.4%	2 ml

7.3 Page gels

1 Prepare the gel mold.

Make sure that the glass plates are clean, wipe them with alcohol. Slightly grease the black rubber spacers, put them in place around the edges of the glass, making sure that the bottom joints are closed with vaseline. Carefully lay the second glass on top and clasp the two plates together. Check that the spacers have not moved. Put the well former in place. Stand the glass plates vertically and fill the mold with distilled water, wait for about 5 minutes to check that there are no leaks, then tip out the water and remove last drops with absorbant paper.

2 Prepare the gel solutions.

The higher the percentage of acrylamide and bis-acrylamide in the gel, the smaller the pores and the slower the run.

The standard gel for isoenzymes contains 7.5% acrylamide.

Make a solution of 22.2% (w/v) acrylamide + 0.6% bis-acrylamide (dissolve the acrylamide before adding the bis).

N.B. Acrylamide is toxic when in powder or in solution.

Take care weighing it and wear gloves when dealing with the gel solution.

Polymerised gels are not toxic.

	35ml soln	35ml soln	30ml soln
	5% gel	7.5% gel	7.5%gel
22.2%acrylamide,	7.9ml	11.8ml	10.1
0.6% bis			
TG buffer (pH8.8)	7.5ml	17.5ml	15.0ml
dist. water	9.5ml	5.5ml	4.7ml

Degas this solution for about 30secs.

Add:

10% Ammonium persulfate	200ul	200ul	171ul
TEMED	5ul	5ul	5ul

Mix gently, use a large syringe or pipette to slowly pour into gel mold. Be careful not to trap bubbles, as these are difficult to remove. Tilt the glass plates slightly to one side when reaching the well-former and continue to fill the mold very slowly, as this helps prevent bubbles being trapped. Fill the mold completely, if the mold is not full the wells will not form properly. A few drops of distilled water may be gently added on top to prevent drying at the edges and to exclude air from the surface. Acrylamide does not polymerise if in contact with air.

Polymerisation is accelerated in the light, so the gel can be put by a window or a lamp to accelerate polymerisation.

3. Storing gels.

Gels may be stored for a day or two before use. The well-former should be kept in place until use.

When the gel has polymerised, the clamps should be removed and the gel can either be completely wrapped in polythene film, or put into place in the electrophoresis apparatus (see below), with the tank buffer in place to prevent desiccation.

7.3.2 Page with a stacking gel

1. Remove clamps and the bottom spacer. Pour 2 litres of tank (electrode) buffer (Tris-Glycine pH8.8) into the bottom half of the apparatus, put the gel-holder in place and push the glass plates + gel into position. Melt a little old 2% agarose gel in the microwave, and use this to seal the joint around the glass and the holder. Check that there are no large bubbles trapped at the bottom of the gel, as this will reduce the flow of current through the gel. To remove these bubbles, lift the holder + gel out of the buffer, then slowly lower it in again, keeping it tilted so the air escapes to one side. If bubbles persist, it may be because there is too much vaseline on the glass, in which case it may be necessary to wipe it off.

2. Carefully remove well-former. Add buffer to the upper part of the apparatus, making sure that the top of the gel is well covered and that there are no leaks. Rinse all the wells with tank buffer using a syringe + needle (this removes any unpolymerised gel).

7.3.3. Preparation of extracts

A spatula-tip of ground lyophilised mycelium (about 5mg) is put into a small Eppendorf tube, 100ml extraction buffer added, mixed with a vortex mixer, then spun down at 14000rpm for 2 minutes. The supernatant can be used immediately or frozen for future use. Very little fungal material is needed for GPI, more is needed for other enzyme systems.

Extraction buffer:

TC 7	800ul
40% sucrose	150ul
Blue juice	50ul

If the lyophilised mycelium was ground with a little (a spatula tip of) sodium metabisulphite, then good results will be obtained with the buffer mix above. If the mycelium was ground without the addition of sodium metabisulphite, it is advisable to add beta mercaptoethanol to the extraction buffer, at a concentration of 0.7%.

7.3.4. Running the extracts

Rinse each well with tank buffer, using a syringe.
Slowly add extracts to wells.

	GPI	PEP
For the 15 well comb	12ul	20ul
For the 25 well comb	8ul	12ul

For the old equipment (thicker gels), run the gel at 10mA current for 30 minutes, then increase the current to 20mA for a further 30 minutes, then increase it to 35mA for the rest of the run. Voltage should start at about 40 -55 V, gradually increasing during the run.

For the new equipment (1mm thick gels), run at 5mA for 30 minutes, then increase to 10mA. If two gels are being run at the same time, double the amperage.

Gels should be run until the blue markers is at the bottom of the gel, or longer if desired.

Staining is carried out as for cellulose acetate gels. Pep activity is normally situated about halfway between the origin and the blue marker, whereas GPI migrates more slowly and is found in the upper part of the gel. Staining solution should be poured quickly and evenly over the appropriate areas.

8. mtDNA HAPLOTYPES

8.1 DNA extraction

1. Harvest mycelium from 8-10 day old pea broth cultures by vacuum filtration. Form the mycelium is a fairly flat shape, put it into 1.5ml Eppendorf tubes, ensuring that air can circulate down to the bottom of the tube. This will speed up the lyophilisation and prevent the drying mycelium from popping up out of the tube.
2. Freeze the mycelium rapidly, then lyophilize for at least 24 hours. Grind in liquid nitrogen with a little sand.
3. Weigh 30-35 mg of ground lyophilized tissue, add 1ml of extraction buffer (microwave first 15-20 seconds) and incubate at 65°C for 1 hour. Once or twice during this time, gently mix the contents by inverting the tubes. Every 15 minutes, mix contents of the tubes by inverting them.
4. Add 333µl of 5M Potassium acetate, shake tube vigorously and put on ice for 20 minutes.
5. Spin tubes at maximum speed for 10 minutes, gently pour supernatant into a new sterile 2ml tube and add 800µl of cold isopropanol. Mix by inversion and put tubes on ice for 30 minutes.
6. Centrifuge for 5 minutes at maximum speed, gently pour off supernatant and dry pellets by inverting tubes on paper towels for about 10 minutes.
7. Resuspend pellet in 100µl of TE buffer.

DNA is of good enough quality now to use, but if you prefer you can continue with a second precipitation. We usually only do up to step 7.

For a 2nd precipitation resuspend pellet in 700µl of TE buffer.

8. Add 75µl of 3M Sodium acetate and 500µl of isopropanol. Mix well by inversion and spin down for 30 seconds.
9. Dump supernatant and wash pellet with 75% ethanol twice, let pellet dry and resuspend in 100µl of TE buffer.
10. DNA quality and concentration should be checked by electrophoresis on an agarose gel.
11. Dilute a part of the stock solution of DNA to 2 ng/µl with TE buffer before PCR.

Buffer	Chemical	Final Concentration
Extraction	EDTA	0.05M
	Tris pH 8.0	0.1M
	NaCl	0.5M
	beta mercaptoethanol	0.7%
	SDS	0.25%

Buffer	Chemical	Final Concentration
TE	Tris HCl pH8	10 mM
	EDTA	1 mM

8.2 DNA electrophoresis

Prepare the following TBE buffer:

Buffer	Chemical	Amount
TBE 5X STOCK	Tris base	54g
	Boric acid	27.5g
	EDTA (pH8)	20 ml of 0.5M stock

Dilute for 3 liters at a time (it's the minimum amount needed for the large gel and buffer tanks), to 0.5 X TBE buffer working solution (0.045 M Tris borate, 0.001M EDTA).

Purpose	Agarose concentration	Amount to Load on gel	Running time
DNA quality	1%	5 μ l	1 hour
PCR products	1%	8 μ l	1 hour
Restriction products	2%	29 μ l	2.5 hours

1. Weigh the corresponding amount of agarose in an Erlenmeyer.
Add TBE buffer.

Gel Size	Ingredients	Agarose Percentage	
		1%	2%
Small	Agarose	0.4g	0.8g
	0.5X TBE	40ml	40ml
Big	Agarose	2g	4g
	0.5X TBE	200ml	200ml

- Heat in microwave until boiling, swirl, and heat again.
- Cool for 5 minutes at 50°C in a water bath and add 1µl of 10mg/ml stock Ethidium bromide per 60ml of solution, swirl to mix. *Whenever dealing with ethidium bromide WEAR GLOVES.*
- Carefully pour the agarose in a levelled gel tray, avoiding bubbles, immediately insert the comb(s), let gel set for 15 minutes.
- Put the gel into the tank and pour TBE buffer over the gel until it is completely immersed. Remove the comb.
- Prepare the samples and load (each mixed with 2µl of bromophenol blue on parafilm or glass) slides.
- Set voltage at 100 for the big gel and 80 for the minigel, and let run:

Purpose	Agarose concentration	Amount to Load on gel	Running time
DNA quality	1%	5 µl	1 hour
PCR products	1%	8 µl	1 hour
Restriction products	2%	29 µl	2.5 hours

DNA with Ethidium bromide can be visualized with UV light of 300nm on the transilluminator. *Wear safety glasses and gloves.*

Dispose of Ethidium Bromide contaminated gel and buffer in the Ethidium Bromide waste bucket.

8.3 Polymerase chain reaction

This method for detecting the different mitochondrial DNA types of *P.infestans* was developed by Gareth Griffiths and David Shaw in Bangor, University of Wales.

8.3.1 Preparation of samples

1. Prepare the following master mix in a stock, without the DNA. Make enough master mix to include a blank sample.
2. Vortex briefly and centrifuge for a few seconds.
3. Dispense 23 μ l of master mix per sample and then add the DNA template to each tube.
4. Centrifuge briefly.

Ingredients of Master Mix	Stock Concentration	Volume / Reaction	Final Concentration
MgCl ₂	25mM	1.5 μ l	1.5 mM
Thermo buffer	10x	2.5 μ l	1 x
dNTP's	1mM	2.5 μ l	100 μ M
Forward Primer	5 μ M	1.6 μ l	0.325 μ M
Reverse Primer	5 μ M	1.6 μ l	0.325 μ M
Taq DNA polymerase	5U/ μ l	0.3 μ l	1.5 units
Water (distilled sterile)		13 μ l	
DNA	2ng/ μ l	2 μ l	4ng

We use a 25 μ l final reaction volume.

8.3.2 Amplification

Perform PCR using the following temperature profiles:

For primer 1:

Purpose	Time	Temperature	Cycles
Denaturation	3 min	94°C	1
Denaturation	30 sec	92°C	35
Primer annealing	30 sec	55°C	

Primer extension	1.10 min	72°C	
Final extension	5 min	72°C	1
Cooling	forever	4°C	

For primers 2 and 3:

Purpose	Time	Temperature	Cycles
Denaturation	3 min	94°C	1
Denaturation	30 sec	92°C	35
Primer annealing	30 sec	62°C	
Primer extension	1.10 min	72°C	
Final extension	5 min	72°C	1
Cooling	forever	4°C	

For operating instructions: read the PCR machine manual.

Check for amplification products on an agarose gel (section 8.2).

8.4 Restriction of PCR products

1. Pipet 10µl of PCR products in clean Eppendorf tubes.
2. Prepare a master-mix; for primer 1 use CfoI, for primer 2 use MspI, for primers 3 and 4 use EcoRI.

Ingredient	Stock Concentration	Volume / Reaction	Final Concentration
Restriction buffer	10 X	3 µl	1 x
Restriction enzyme	10U/µl	0.1 µl	1 unit
Distilled water		16.9 µl	

3. Vortex and centrifuge 2 seconds.
4. Add 20µl of the mastermix to the DNA samples, vortex and centrifuge the samples 2 seconds at full speed, incubate for 1 hour at 37°C in a water bath.
5. Add 3µl of bromophenol blue to the samples and load the restriction products on a 2% agarose gel (see section 8.2).

With CfoI it is possible to discriminate between Iia (1118 bp fragment) and Ia, Ib and Iib (907, 211 bp fragments).

With MspI it is possible to discriminate between Ib (641, 350, 79bp fragments), Ia, Iib (720, 350 bp fragments) and Iia (720,203,147 bp fragments).

With EcoRI and primer 3 it is possible to discriminate between Ia, Ib (1064, 228 bp fragments) and Iia, Iib (1292 bp fragment) isolates.

With EcoRI and primer 4 it is possible to discriminate between Ia, Ib (287,361,209 bp fragments) and Iia , Iib (596, 361 bp fragments).

9. Ribosomal ITS region amplification

This is a PCR method developed by Paul Tooley et al 1997 (Appl. Env.Microbiol. 63,1467 -1475) for the early detection of *P.infestans* in tubers. The primers used (ITS3: 5'-GCATCGATGAAGAACGCAGC-3' and PINF2: 5'CGATTCAAATGCCAAGCTAAAG -3' have been specifically designed to detect *P.infestans*, although they do also amplify *P.phaseoli* and *P.mirabilis*. PCR with these primers produces a single 456bp product with these species. We have not yet used it for detection, but have used it successfully on purified DNA, as a check to see whether isolates from wild Solanaceae were *P.infestans*.

1. Extract DNA as indicated in section 8.1, dilute to 10ng per μl with TE.
2. Make up the following master mix, multiplied by 1.1x the number of samples + 1 control without DNA:

x 10 buffer		2 μl
25mM MgCl ₂	1.8mM	1.44 μl
2.5mM dNTP mix	100 μM	0.8 μl
10 μM primers ITS3	0.1 μM	0.2 μl
PINF2	0.1 μM	0.2 μl
taq		0.07 μl
H ₂ O		14.29 μl
10ng/ μl DNA		1 μl
Total		20 μl

3. Dispense 14 μ l into each tube and add 1 μ l of DNA extract.
4. Run the following amplification programme:

94°	1 minute	1 cycle
94°	15 secs	
50°	1 minute	30 cycles
72°	45 secs	
72°	5 minutes	1 cycle

These times are longer than specified in Tooley et al. because they are also suitable for several anchored microsatellite primers, which can be used for amplification at the same time.

5. Load 10 μ l on a 1.4% or 2% gel, alongside a DNA ladder, as explained in Section 8 and view after 1.5 hours.

10. POTATO DNA EXTRACTION

1. Weigh 500mg of fresh leaf tissue, grind with liquid Nitrogen , 250mg of beta-metabisulfite, and sand.
2. Add 2.5ml extraction buffer, keep on ice while adding to other samples.

Buffer	Ingredient	Concentration
Extraction pH 8.2	Sorbitol	350 mM
	Tris	100mM
	EDTA	5mM
Lysis	Tris	200mM
	NaCl	2M
	CTAB	2%
	EDTA	50mM

3. Add 2.5ml of lysis buffer (preheated to 60°C and 2%CTAB added just prior to use).
4. Add 100µl of 10% sarkosyl and then incubate tubes for 40 minutes at 65°C. Invert tubes from time to time.
5. Add 5ml of chloroform and mix gently, and centrifuge at 10000 rpm for 10 minutes.
6. Transfer aqueous supernatant to a new tube and add 1 ml of cold isopropanol. Mix by inversion and leave in fridge. Yield may also be increased by adding 100% ethanol.
7. Centrifuge at 10000 rpm for 5 minutes.
8. Remove isopropanol and rinse with 70% alcohol, let air dry and add 500µl of TE.
9. Treat with 1µl of 5mg/ml of RNase for 30 minutes at 60°C.

NOTE: these method is also good for isolating *P. infestans* DNA, just use 1/5 of everything.