PROTOCOLS FOR FUNGICIDE SENSITIVITY ASSAY USING DETACHED LEAF AND DNA EXTRACTION FOR PHYTOPHTHORA INFESTANS

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Contents

Protocols to be used in Asiablight training

Late blight, caused by *Phytophthora infestans*, is a worldwide problem that is still mainly controlled by repeated prophylactic use of fungicides throughout the growing season. Effective blight management strategies rely on knowledge of the efficacy of available fungicides to control contemporary genotypes of *P. infestans*. The testing for the sensitivity of *Phytophthora infestans* to fungicides can be conducted either under greenhouse conditions or by using standard laboratory techniques such as media amended with different concentrations of fungicides, floating leaflet disks on fungicide suspensions, use of tuber disks and detached-leaf assay. Currently, the detached leaf technique yields the most reliable assay of *P. infestans* sensitivity to the fungicides and is the recommended assay for determining the fungicide sensitivity.

The protocols for the detached leaf assay described below are routinely followed at Prof. Sanjoy Guha Roy's laboratory at the Department of Botany, West Bengal State University, Barasat, Kolkata 700126, India, which is in a tropical environment. The protocol has been simplified and modified for Asiablight to enable regional representatives from diverse regions to replicate the procedure without being dependent on external resources to establish baseline sensitivity/update information for their regions/countries. Similarly, the other protocols shared here, such as DNA extraction from the mycelium of *Phytophthora* spp., are also routinely followed in the laboratory and have been well tested.

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A. Protocol for the assessment of fungicide sensitivity by detached leaf assay technique for late blight disease caused by *Phytophthora infestans*

A. Materials and Reagents

1. Glass beaker 250 ml
2. Cheese cloth
3. Petri Dish (100 mm × 15 mm)
4. Parafilm (PM-996)
5. Heavy-duty paper towels
6. *P. infestans* isolate
7. Sterile water
8. 70% ethanol
9. A pair of Scissors
10. Scalpel
11. Forceps 4''.
12. Spirit lamp
13. Haemocytometer
14. Glass slide
15. Cover slips
16. Micropipette (20ul, 200 ul, 1000 ul)
17. Micro tips

18. Microfuge tubes
19. Conical 250 ml
20. Micro pestle
21. Camera
22. Fungicide (Mancozeb 75%)

B. Equipment

1. Laminar airflow
2. Light microscope with 10x and 40x objectives
3. 4 °C refrigerator / Cold room
4. Plant growth chamber /Incubator retrofitted with lighting arrangement (photoperiod 16/8)
5. 10 × 10 cm pots
6. 15 × 15 cm pots
7. Weighing balance
8. Centrifuge
9. Magnetic stirrer
10. Vortex

C. Software

1. ImageJ
2. Microsoft Excel
3. R

D. Procedure

1. Preparation of Inoculum

- **Inoculum Source:** Either directly from freshly infected LB leaves or from previously isolated/referenced *P. infestans* isolate/(s)

a. Directly from freshly infected leaves:

(i) Harvest 1-2 freshly infected leaflets (ideally lateral leaflets of the same petiole, alternatively from the neighbouring petiole of the same plant) having a single lesion covering half of the leaf. Prepare a duplicate set also.

(ii) Prepare a moist chamber by using disposable sterile petri plates/ sterile glass petriplates: Open a fresh sterile petriplate under the laminar airflow, place an autoclaved blotting paper/ handmade paper cut to size of the petri plate into the petriplate, pipette just enough autoclaved water to wet the paper making sure that the water does not accumulate above the paper, it should be just about wet.

(iii) Carefully place the infected leaflets ABAXIAL side up in the moist chamber.

(iv) Cover the upper lid and seal the petriplate with parafilm.

(v) Since these steps would be done during the potato growing season, there is no need to put the moist chamber in an incubator if the room temperature is around 16-20 °C and there is enough natural daylight in the laboratory. The moist chamber can also be placed near the windows for daylight. However, if these conditions are not met, place the specimen in an incubator at 18 °C with a 16/8 photoperiod.

(vi) Remove the lid of the petriplate after 24 hours when enhanced mycelial growth can be seen.

(vii) Carefully remove the leaflets from the moist chambers and place them in a new disposable sterile petriplate with the ADAXIAL side up, such that the mycelial growth touches the floor of the petriplate. Repeat this separately for the duplicate set.

(viii) Flood the floor of the petriplate with about 1.5 ml of autoclaved water, such that the water level touches the mycelial mass on the leaf. Repeat this separately for the duplicate set.

(ix) Hold the petiolule and gently wash the leaflet by moving it sideways on both sides, while the mycelial side remains submerged to release the sporangia from the sporangiophores. Repeat this separately for the duplicate set.

(x) Repeat this step for the other leaflet. Repeat this separately for the duplicate set.

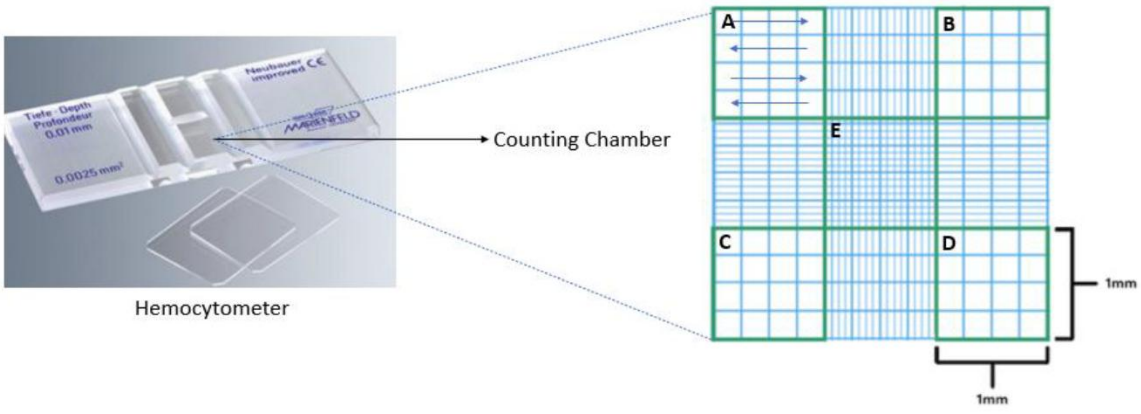
(xi) Pool all the water from set 1, which now has the sporangia, into an Eppendorf, keeping the leaves aside in the petriplates. Do not discard the leaves for now; you might need to do a 2nd wash cycle. Repeat this separately for the duplicate set.

(xi) For the other set, do not process immediately; wait to see the sporangial concentration reached in the next step. If the concentration is too low (which you will determine in the next step), reuse the first set. Alternatively, repeat the steps to utilise the second set as needed to reach the desired concentration.

b. From previously isolated cultures:

Ideally, a selected set of isolates representing the different genotypes from different locations should be available for each region/country. (For details, see Andrivon 2011; and Lynott 2024) which is to be maintained on a suitable growth medium, such as rye A media, Pea agar media at a controlled temperature (e.g., 18°C).

- **Concentration determination and Zoospores extraction** From the Eppendorf with the harvested sporangia, take 10 µl of the suspension and place it in one block of the haemocytometer, place a cover slip on it and check under a light microscope (10X). The following steps need to be followed:



Hemocytometer

a. Moisten the shoulders of the hemocytometer and gently press the cover slip into place.

b. Ensure that the cover slip is properly seated on the surface of the counting chamber. When the two glass surfaces make correct contact, Newton's rings will be visible.

c. Apply 10 µl of sporangium suspension at the edge of the cover slip so that it is drawn into the chamber by capillary action, completely filling the chamber.

d. Make sure the suspension is thoroughly mixed beforehand, either by gently agitating the tube containing it or by using a sterilized pipette by drawing and dispensing suspension two to three times in the same tube.

e. Place the hemocytometer under the microscope using the 10× objective to locate the grid.

f. Allow the spores to settle in the chamber for about two minutes before counting.

g. Then focus on one of the large squares at each corner, each of which is subdivided into 16 smaller squares.

h. Using a hand tally counter, record the number of spores in each large square (A, B, C, D, E).

i. Count the spores within each small square as well as those touching the right-hand or bottom boundary lines, but exclude any spores on the left-hand or top boundary lines.

10. Concentration (spores/mL) = **average count per square** × **dilution factor** × **volume conversion factor.**

For a standard Neubauer haemocytometer, the volume conversion factor is ***10^4*** *when you use the commonly counted large squares*

Example: Suppose you counted spores in 4 large squares and obtained these counts:

- Square A: 28
- Square B: 33
- Square C: 31
- Square D: 28
- Square E: 20
- Sum the counts: 28 + 33 + 31 + 28 +30 = 150.
- Number of squares counted = 5.
- Average count per square = sum / number = 150 ÷ 5 = 30.
- *Suppose the sample was not diluted before counting — that gives a dilution factor of 1.*
- Using the conversion factor 10^4 (i.e., 10,000), concentration = average × dilution factor × 10,000.

Now compute digit-by-digit:

- average × dilution factor = 30 × 1 = 30.
- 30 × 10,000 = 3,00,000.

So the calculated concentration = ***3,00,000 spores per mL*** *(3.0 × 105 spores/mL).*

(ii) Check the concentration, at a minimum, it should be in the range of 104 sporangia/mL, and ideally it should be 5 × 104 sporangia/mL (i.e. 50,000 sporangia per mL). If the concentration is low, process the duplicate set in the same manner. If the concentration is high, adjust the concentration with water accordingly.

(iii) Once the concentration is standardised, for zoospores extraction, keep the Eppendorf at 4 °C for 4 hrs to release zoospores.

2. Leaf Sample Collection and Setup

Potato plants of susceptible cultivars (will vary with region/country) are to be grown in pots from seed tubers maintained under glasshouse conditions. No pesticides are to be applied. When plants were approximately 6 weeks old, the middle leaflets are to be harvested for use immediately before inoculation. Use a 0.5% hypochlorite solution for surface disinfection.

Follow the following methods for surface disinfection:

a. Dilute commercial bleach (usually 4–6% NaOCl) with sterile distilled water to the target concentration (commonly 0.5 available chlorine).
b. Rinse leaves under running tap water to remove loose debris.
c. Immerse leaves in the prepared 0.5% sodium hypochlorite solution.
d. Gently agitate to ensure all surfaces are in contact with the disinfectant.
e. Typical exposure time is 30s-1 minutes for potato leaves.
f. After disinfection, rinse leaves thoroughly 3–5 times with sterile distilled water to remove residual chlorine. (Gentle agitation during rinses helps remove traces of bleach that can damage tissue or inhibit the assays.)
g. Transfer the sterilised leaves to a sterile moist chamber in a laminar-flow hood.

- **Leaf Setup:**

Place the excised, disinfected leaves in a water-saturated moist chamber (abaxial side up) to maintain humidity.

3. Inoculation and Incubation

- **Inoculation:** Pipette 10 µl of the zoospore suspension onto the abaxial (lower) side of each fungicide-treated and control leaflet set.
- **Controls:** For comparison, include leaflets inoculated with sterile deionised water (for negative control) and fungicide alone.
- **Disease Development:** Incubate the inoculated leaves for 5 to 8 days at 18°C in a 16/8 hrs light and dark period.

4. Disease Scoring

- **Scoring:**

After the incubation period, assess disease severity on the treated leaves.

- **Measurements:**

You can score the severity of late blight in several ways:

- **Lesion size:** Measure the diameter of the blighted lesions.
- **Area:** Use image analysis software (Image J) to measure the total diseased leaf area or the percentage of the total leaf area that is infected.

To take pictures, follow the following steps:

a. Infected leaves need to be laid on a flat and contrasting background (Preferably white).
b. *Include a Ruler/Graph paper in the same plane as the leaves (both leaves and scale should come in one frame).*
c. Photograph at high resolution; save as TIFF or PNG (preferred for accuracy).

Image J user guide for detached leaf assay:

d. *Install the ImageJ software in the computer system (https://fiji.sc/). Launch ImageJ.exe (for Windows)*
e. *Go to **File → Open…** and load infected leaf image.*

Calibrate the scale:

f. *Select the **Straight Line Tool**.*
g. *Draw a line exactly along a known distance based on ruler/graph sheet (e.g., 1 cm on the ruler/graph).*
h. *Go to **Analyse → Set Scale…**.*
i. Enter the known distance (e.g., 1 cm) and units (cm or mm).
j. *Click **OK**. (Now all measurements will be in actual units.)*

Convert and prepare the image

k. *If the image is in colour:* ***Image → Type → 8-bit*** *(grayscale).*

l. *Adjust contrast:* ***Image → Adjust → Brightness/Contrast*** *so leaves are clearly visible.*

m. *Threshold the image:* ***Image → Adjust → Threshold…***

n. Move sliders until the leaves turn red while the background stays clear.

o. *Click* ***Apply*** *to create a binary image of just the leaves.*

Analyse leaf area

p. *Ensure the entire infected area of a leaf (or each leaf) is selected.*

q. *For single leaf: use the* ***Wand Tool*** *or* ***Freehand Selection Tool*** *to select.*

r. *Check "Display results" and "Summarise" to get the total area.*

s. *Click* ***OK*** *— ImageJ generates a table with the area of each leaf and/or the total area.*

Save results

t. *In the* ***Results*** *window:* ***File → Save As…*** *(CSV or Excel).*

u. *Save processed images:* ***File → Save As → TIFF/PNG*** *to keep a record of thresholding.*

5. Data Analysis

- **Comparison:**

Compare the disease development on leaves treated with different fungicide concentrations against the control.

- **Baseline Sensitivity:**

Compare the sensitivity of the isolate to a baseline sensitivity profile to determine if it is less sensitive or resistant to the fungicide.

Baseline sensitivity is the reference level of susceptibility of a fungus/oomycete to a given active ingredient, measured on isolates collected from areas or seasons where that fungicide has not yet been used.

EC50 calculation following Alexander *et. al.*, 1999.

Example:

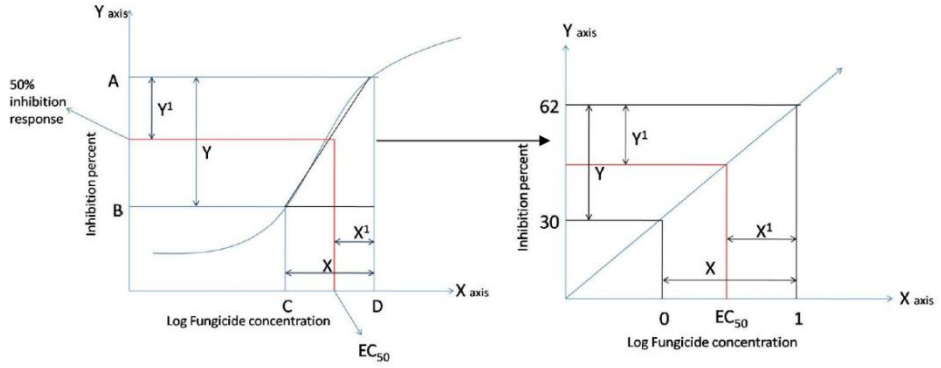
Fungicide concentration	Log fungicide concentration	Percent inhibition
0.1	-1	10.0
0.5	-0.301	17.75
1	0	30.0
10	1	62.0
100	2	100

Relative EC50 calculation:

$$\text{Relative 50\% inhibition} = \frac{\text{Minimum inhibition percent at lowest fungicide concentration tested} + \text{Maximum inhibition percent at highest fungicide concentration tested}}{2}$$

$$= \frac{10+100}{2}$$

$$= 55$$



Where , A = 62 , B = 30 and C = 0 , D = 1

$$\frac{Y^1}{Y} = \frac{X^1}{X} \quad \text{----------} \quad 1$$

$$\text{Therefore,} \quad X^1 = \frac{Y^1}{Y} * X \quad \text{----------} \quad 2$$

$$EC\ 50 = C + (X - X^1) \text{----------} \quad 3$$

$$\text{Or,}\ EC\ 50 = D - X^1 \text{----------} \quad 4$$

$$X = D - C \text{ --------- Dosage interval----------} \quad 5$$

Y = Interval between A and B
= (A – B)

Y^1 = Interval between 50% inhibition and inhibition percent of next highest concentration
= (A – 50% inhibition)

$$\text{From equation 2 ,} \quad X^1 = \frac{Y^1}{Y} * X$$

$$\text{Or,}\ X^1 = \frac{(A - 50\%\ \text{inhibition})}{Y} * X$$

$$\text{From equation 4 ,} \quad EC_{50} = D - X^1$$

$$\text{Or,}\ EC_{50} = D - \frac{(A - 50\%\ \text{inhibition})}{Y} * X$$

$$= 1 - \frac{(62 - 55)}{(A - B)} * (D - C)$$

$$= 1 - \frac{(62 - 55)}{(62 - 30)} * (1 - 0)$$

$$= 0.781$$

Anti log of 0.781 = 6.042

Therefore, EC_{50} = 6.042 ug/ ml

Preparation of the Excel sheet to automate the process*

In Excel, make each column in the following way: *(A Sample Excel sheet has also been prepared with the formula, which can be easily used and is being distributed with this protocol)

1. Calculate inhibition percentage for each concentration = $\left(\frac{\text{Control - Treated}}{\text{Control}} \text{ X } 100 \right)$

2. Take the minimum response value in one column and the maximum response value in another column.

3. Calculate 50 % response value = $\left(\frac{\text{Minimum response value + Maximum response value}}{2} \right)$

4. Take the immediate nearest minimum response value to the 50 % response value in one column and take the immediate nearest maximum response value to the 50 % response value in another column.

5. Take the corresponding concentration of the nearest minimum response value in one column and take the corresponding concentration of the nearest maximum response value in another column.

6. EC 50= 10 ^ (Log10 (Nearest minimum concentration) + (50% response – Nearest minimum response) / (Nearest maximum response – Nearest minimum response) x Log10 (Nearest maximum concentration / Nearest minimum concentration))

Further References

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B. Protocol for the extraction of DNA from the mycelium of Phytophthora spp

1. Take approximately 0.1 g of mycelium in a sterile 1.5 ml microcentrifuge tube.
2. Add 750 µl of DNA extraction buffer.
3. Thoroughly grind the mixture using a sterile micro pestle for at least 4-5 minutes, then centrifuge at 13000 rpm for 5 minutes.
4. Transfer the aqueous phase to a new sterile 1.5 ml microfuge tube.
5. Add an equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1) and gently invert the tube for mixing.
6. Centrifuge the mixture at 13000 rpm for 5 minutes.
7. Carefully transfer the supernatant above the protein layer to a new sterile microfuge tube.
8. Add 2 µl of RNase A and incubate at 37°C for 60 minutes.
9. Add 1 ml of ice-cold isopropanol and incubate at -20°C overnight (alternatively for a few hours)
10. Gently invert the tube 5-7 times to mix, then centrifuge at 13000 rpm for 10 minutes.
11. After centrifugation, observe a small whitish pellet at the bottom; quickly drain off all the aqueous solution.
12. Add 1 ml of 70% ethanol to the pellet and centrifuge for 2 minutes at 13,000 rpm (repeat this step twice).
13. Air-dry the pellet briefly and finally resuspend it in 100 µl of molecular biology-grade water.

Table 1. Composition of stock and working concentration of DNA extraction buffer.

Chemicals	Stock Concentration	Working Concentration
Tris-HCl (pH 7.5)	1 M	200 mM
Sodium chloride	5 M	250 mM
EDTA (pH-8)	0.5 M	25 mM
SDS	10%	0.5%

Table 2. EC50 Calculation Example. ***(Authored by Chen, Han).***

	Control	Test Conc. (ppm)	Test Conc. (ppm)	Test Conc. (ppm)	Test Conc. (ppm)	Test Conc. (ppm)	Test Conc. (ppm)	Test Conc. (ppm)														
Sample	0	0.0001	0.001	0.01	0.05	0.075	0.1	1	Inhibition percentage at 0.0001	Inhibition percentage at 0.001	Inhibition percentage at 0.01	Inhibition percentage 0.05	Inhibition percentage at 0.075	Inhibition percentage 0.1	Inhibition percentage at 1	Minimum response	Maximum response	50% Response	Nearest minimum response	Nearest maximum response	Nearest minimum concentration	Nearest maximum concentration	EC 50
P melonis	5.6	5.5	5.4	5.3	0.3	0.2	0.1	0	1.785714286	3.571428571	5.357142857	94.64285714	96.42857143	98.21428571	100	1.785714286	100	50.89285714	5.357142857	94.64285714	0.01	0.05	0.022723473

Inhibition percentage =(Control-Treated)/Control*100

50% Response =(Minimum response+Maximum response)/2

EC 50 =10^(LOG10(Nearest Minimum Concentration)+(50% Response-Nearest minimum response)/(Nearest maximum response-Nearest minimum response)*LOG10(Nearest maximum response/Nearest minimum response))

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