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Phytophthora infestans (Late blight) Infection Assay in a Detached Leaf of Potato

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[Abstract] *Phytophthora infestans* is a hemibiotroph oomycete that primarily infects potato and tomato. It infects stems, leaves, and tubers and fruits of potato and tomato. High throughput and reproducible infection assays are prerequisites to find sources of resistance in any crop. In this protocol, we describe a detached leaf assay (DLA) for conducting the virulence assay of *P. infestans* in potato leaves. A late blight infection assay using a potato detached leaf is a semi-high throughput assay in which hundreds of plants can be screened in a laboratory setting.

Keywords: *Phytophthora infestans*, Potato, Detached leaf assay, Late blight

[Background] Potato (*Solanum tuberosum* L.) is one of the most important non-cereal food crops in terms of food and nutritional value (Zhang *et al.*, 2017). Late blight of potato caused by the oomycete pathogen *P. infestans* is one of the most devastating potato diseases in the world and is the most important yield-limiting factor in potato production (Haverkort *et al.*, 2008 and 2016; Fisher *et al.*, 2012). Breeding for late blight resistance is considered an important factor to fight against this disease. For this purpose, identification of novel sources of resistance in the available germplasm is a crucial step. Several testing methods such as field tests, whole plant assays, and detached leaf assays (DLA) have been developed. DLA provides increased infection and potato leaves showing more susceptibility to *P. infestans* than the field and whole plant assays (Stewart, 1990; Vleeshouwers *et al.*, 1999; Vossen *et al.*, 2016). Since the genotype of the host and pathogen are generally static in infection assays, observed differences in susceptibility among testing methods are likely due to variation in environmental conditions. DLA is suited for identification of qualitative resistance available in the germplasm which is typically a qualitative trait governed by a single or a few disease resistant (R) genes.

Materials and Reagents

1. Conical tubes
2. Combitips advanced 1.0 ml (Eppendorf, catalog number: 0030089430)
3. Cheese cloth
4. Petri Dish (100 mm × 15 mm) (Fisher Scientific, catalog number: FB0875713)
5. Parafilm (PM-996)
6. Nunc square standard height bioassay dishes (Thermo Fisher Scientific™, catalog number: 240835)
7. Heavy-duty paper towels (Uline, catalog number: S-13631BLU)

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8. Spreader (Universal Medical, catalog number: HS86655)
9. *P. infestans* isolate, US-23
10. Sterile water
11. 70% ethanol
12. Potato genotypes, obtained from U.S. Potato GenBank (Sturgeon Bay, WI)
13. Fertilizer (Peters' Professional 20-10-20 Peat Lite special)
14. Growing media
    a. For *P. infestans* Rye A media (Caten and Jinks, 1968) (60 g of Rye grain, 15 g of agar and 20 g of sucrose for 1 L of distilled water, for detail please see Reference)
    b. For plants soil mixtures (All-purpose mix BM1, Berger)

Equipment

1. Eppendorf Repeater E3 (Eppendorf, catalog number: 4987000398)
2. Biological safety cabinet
3. Light microscope
4. Scanner (Epson, model: Perfection V700 Photo)
5. Scalpel
6. Hemocytometer
7. Lamp
8. Cold room at 4 °C or refrigerator
9. Incubator (Fisher Scientific, catalog number: 97-990E)
10. 10 × 10 cm pots
11. 15 × 15 cm pots

Software

1. ImageJ

Procedure

A. Growing conditions of plants and collection of leaves
   1. Germinate potato seeds or plant tubers, cuttings or tissue culture plantlets in a greenhouse in soil-less potting mix (BM1, Berger) in a 10 × 10 cm pot.
   2. Transplant the seedlings after two weeks into the 15 × 15 cm pots.
   3. Maintain a temperature of 22 °C during the day (a photoperiod of 17.5 h) and 20 °C during the night (dark) in the greenhouse.

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4. To maintain the health of plants, irrigate regularly however avoid excessive moisture, fertilize once a week and spray against insects and foliar disease, when necessary. However, avoid spraying with fungicides for one week before inoculations.

B. Growing conditions of *P. infestans*

1. Grow *P. infestans* isolate US-23 on a Rye A media plate (Figure 1) at 18 °C. This plate can be used as a master plate for next 3 months for the further sub-culture.

Figure 1. *P. infestans* growth in Rye A plates after 12 days

2. Cut three plugs (5 mm) of *P. infestans* isolate, US-23 from Rye A media.
3. Place these three plugs in a new Rye A media plate in a triangular fashion (Figure 1).
4. Seal the plates properly with parafilm and incubate in the dark at 18 °C.
5. Keep the plates facing downward to avoid any moisture development on the plugs.

C. Inoculum preparation

1. Harvest the sporangia from a 10-14-day old Rye A cultured plate (Figure 1) by flooding the plate with 5 ml ice-cold sterilized water (to expedite the release of zoospores) and mix properly with a spreader.
2. Keep the plate at 4 °C for 2 to 4 h to release zoospores.
3. Harvest the zoospores by filtering the liquid from each plate through two layers of cheesecloth (to remove mycelia) and dilute in 20 ml of ice-cold sterilized water.
4. Count the motile zoospores using a hemocytometer under a microscope and adjust the concentration to 50,000 zoospores per ml of water. Generally, we make 100 ml final solution (zoospores and water) with 50,00 zoospores from a 10-14-day old Rye cultured plate.

D. Infection assay

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1. Collect healthy, full-grown compound leaves having at least three leaflets from 5-8-week-old plants if available. Care should be taken not to collect old (start showing sign of yellowing) or very young leaves (not-fully developed).
2. Set up a bioassay plate lined with wet Uline heavy-duty paper towels (Figure 2).

Figure 2. Late blight infection assay conducted on bioassay plate lined with wet paper towel. Top cover was removed to take the picture.

3. Drop Inoculate the abaxial side of each leaflet with 10 µl droplets (4 to 6 droplets per leaflet) of inoculum (50,000 zoospores per ml) with an Eppendorf repeater.
4. Keep the bioassay plates in a room with natural light at a temperature of 21 °C.

E. Symptom monitoring

1. The first symptoms can be observed 3 days after inoculation as black/brown lesions, sporulation, and a water-soaked area at the point of pathogen inoculation.
2. Subsequently, symptoms enlarge and cover the whole leaf in the case of highly susceptible genotypes after 5 days.
3. Assess the leaves visually for the appearance of symptoms using a 1-9 scale (Karki *et al.*, 2020) (Figure 3) or quantified by using ImageJ software (Rueden *et al.*, 2017), 5 days after inoculation.

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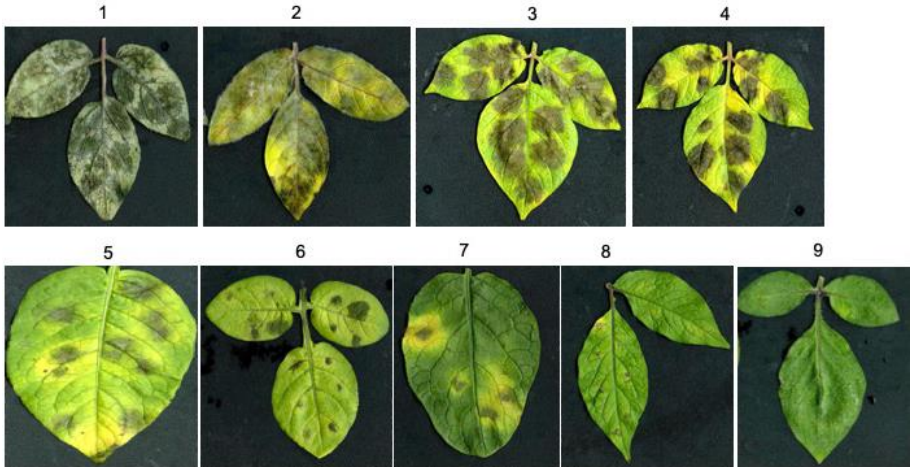

Figure 3. The 1-9 scale used to evaluate the late blight infection. The mean leaf area covered with blackish/brown lesions, sporulation, and water-soaking was calculated using ImageJ and assigned a value based on this area. 1 = ≥90%, 2 = 81-90%, 3 = 71-80%, 4 = 61-70%, 5 = 41-60%, 6 = 21-40%, 7 = 10-20% with cell death at the point of inoculation, 8 = ≤10% with cell death at the point of inoculation, and 9 = 0% infection, no visible symptoms, clean leaves.

F. Quantification of diseased area

1. Obtain a digital image of the leaf using a flatbed scanner or camera (Image type: 24-bit color, Resolution: 300 dpi).
2. Start ImageJ and open the image (File > Open…).
3. Select the ‘Straight Line’ tool.
4. Draw a line to an object of known size (*e.g.*, coin or ruler).
5. Go to ‘Analyze > Set scale and enter the preferred unit of length (*e.g.*, cm, mm, or inches) and the known distance of the line from Step 4. Click OK.
6. Use the ‘Paintbrush tool’ to highlight the diseased area of the leaf. Double-click the paintbrush icon to set the brush options, including brush width. Care should be taken to select only the diseased area and not other injuries.
7. Go to ‘Image > Type > 8 bit’.
8. Go to ‘Image > Adjust >Threshold (to get red leaf images).
9. Select the dark background checkbox, and slide both bars to the very right (255 value). The selected infected area should be red and the remainder of the leaf photo should be in greyscale.
10. Close the threshold settings window.
11. Use ‘Freehand selection’ tool and trace the outline of the whole leaf area.
12. Go to ‘Analyze > Set Measurement’ and select only the ‘Area’ and ‘Area fraction’ checkboxes, deselect any other checkboxes, and click OK.
13. Go to ‘Analyze > Measure’ or press Ctrl+M to measure the diseased leaf area. A separate ‘Results Window’ will pop up showing the whole area and the % area, which represent the

surface area of the outlined leaf in square units defined in step 5 and the percent infected area of the leaf, respectively.

14. When closing the 'Results Window', you will have the option to save the results.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

This protocol describes details of the late blight infection assay used in experiments previously published by Karki *et al.* (2020). Work was supported by the USDA NIFA/NSF Plant Biotic Interactions Program award number: 2018-67014-28488.

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