

Hyaloperonospora arabidopsidis* (Downy Mildew) Infection Assay in *Arabidopsis

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[Abstract] *Hyaloperonospora arabidopsidis* (*Hpa*; formerly *Peronospora parasitica* or *Hyaloperonospora parasitica*) is an oomycete downy mildew pathogen of the model plant *Arabidopsis*. The pathosystem between *Arabidopsis* and *Hpa* has been extensively used to study host/pathogen co-evolution (Coates and Beynon, 2010). As *Hpa* is an obligate biotrophic pathogen, its host is absolutely required for survival. Thus, *Hpa* must be maintained on susceptible *Arabidopsis* accessions and mutants. Growth of *Hpa* is evaluated in two ways; counting conidiospores (Asai *et al.*, 2014) or counting sporangiophores after trypan blue staining (Holt *et al.*, 2005). Here, we describe how to do inoculation with *Hpa* and how to evaluate *Hpa* growth on *Arabidopsis*.

Materials and Reagents

1. Conical tubes (50 ml) (BD Biosciences, Falcon®, catalog number: 352070)
2. Miracloth (Calbiochem®, catalog number: 475855)
Note: Currently, it is "EMD Millipore Corporation, catalog number: 475855".
3. Labeling Tape (Shamrock, catalog number: ST20)
4. Paper towels
5. *Arabidopsis* lines and *Hpa* isolate. Ws-2 *eds1-1* mutants [the accession previously reported as Ws-0 is in fact Ws-2 (Parker *et al.*, 1996)] and Col-0 plants were used as susceptible and resistant accessions of *Arabidopsis* for *Hpa* isolate Emoy2, respectively.
6. Ethanol (70%)
7. Plastic tray (270 x 270 x 60 mm) with a transparent lid
8. Sterile water
9. Bright-Line™ Hemacytometer (Sigma-Aldrich, catalog number: Z359629)
10. Trypan blue (Sigma-Aldrich, catalog number: T6146)
11. Phenol (Wako Pure Chemical Industries, Siyaku, catalog number: 160-12725)
12. Chloral hydrate (Sigma-Aldrich, catalog number: C8383)
13. Glycerol (Wako Pure Chemical Industries, Siyaku, catalog number: 07500616)
14. Trypan blue solution (see Recipes)
15. Chloral hydrate solution (see Recipes)

Equipment

1. Scissors
2. Biological safety cabinet (Labconco, model: Purifier Delta Series Class II Type A2 Cabinet)
3. Airbrush Kit (Airtex, model: ASCF4 and KIDS105)
4. Growth chamber (NKsystem) (Nippon Medical & Chemical Instruments Co., catalog number: LPH410S)
5. Weight (Sartorius, model: Quintix 224-1S)
6. Water bath (NE1-8) (Thermo Fisher Scientific, catalog number: 11459499)
7. Labo shaker (BIO CRAFT, catalog number: BC730)
8. Stereomicroscope (Leica Microsystems, catalog number: M165FC)

Procedure

A. Inoculation with *Hpa*

1. Sterilize scissors and inside of the biological safety cabinet using 70% ethanol.
2. Open a plastic tray with a transparent lid containing *Arabidopsis* plants that are densely covered by sporangiophores (Figure 1A) in biological safety cabinet to avoid diffusion of conidiospores.

Note: To check contamination of different isolate(s) of Hpa, using both susceptible and resistant Arabidopsis accessions for propagation of Hpa is recommended (Figure 1A).

3. Harvest aerial parts of the *Arabidopsis* plants in a 50 ml conical tube (Figure 1B).

Note: Fill the tissues up to 20 ml marker on the tube (see Figure 1B). Avoid any soil contaminations because it may cause propagation of soil inhabiting pathogens.

4. Put 15 ml sterile water in the 50 ml conical tube and shake it gently several times to obtain sporangia in water.
5. Filter the obtained suspension using Miracloth (Figure 1C).
6. Measure the concentration of conidiospores in the suspension using a hemacytometer (Figure 1D) and dilute with water to a concentration of 5×10^4 conidiospores per ml water.

Note: To know how to count with hemacytometer, check the link (<http://www.hemocytometer.org/>).

7. Saturate *Arabidopsis* plants with the suspension by spraying using Airbrush Kit.

Note: Usually, 2-week-old plants and 5-day-old seedlings are used for evaluation of Hpa growth by counting conidiospores and counting sporangiophores, respectively. 2-week-old plants are used for propagation of Hpa. Arabidopsis plants are grown at 22 °C and 60% humidity under a 10 h photoperiod in environmentally controlled growth cabinets.

8. Place the inoculated plants in a plastic tray with a transparent lid to maintain high

humidity (90-100%) conditions (Figure 1E).

Note: High humidity is very important for growth of Hpa. Thus, sealing off a plastic tray with a transparent lid using Labeling Tape is recommended.

9. Incubate the *Hpa*-inoculated plants in a growth chamber at 16 °C under a 10 h photoperiod until the day of sampling.

Note: Timing of sampling depends on the combinations of Hpa isolates and Arabidopsis accessions. Usually, from 4 to 7 days after the inoculation would be the proper timing.

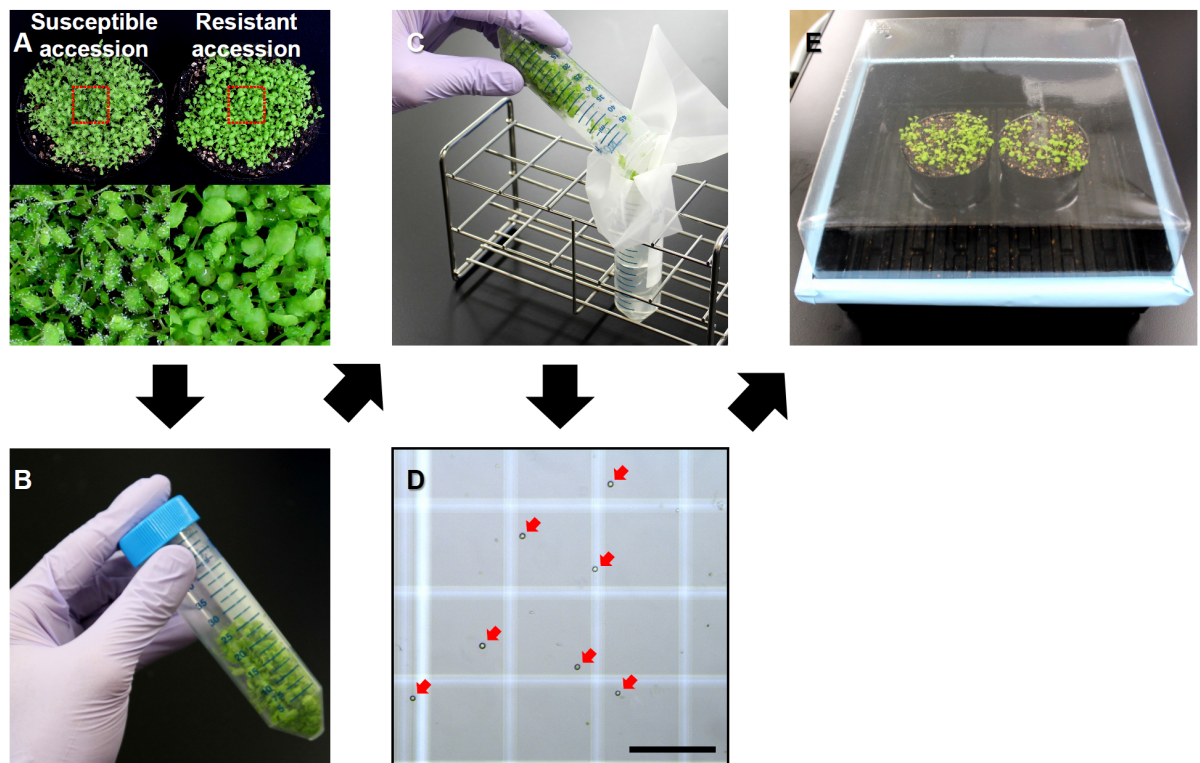


Figure 1. Procedure of inoculation with *Hpa*. A. Susceptible and resistant accessions of *Arabidopsis* 7 days after inoculation with *Hpa*. Ws-2 *eds1-1* mutants (the accession previously reported as Ws-0 is in fact Ws-2; (Parker et al., 1996) and Col-0 plants were used as susceptible and resistant accessions of *Arabidopsis* for *Hpa* isolate Emoy2, respectively. Red boxes: Close-up shown in lower photographs. B. *Arabidopsis* plants harvested in 50 ml conical tube. C. The illustration of filtering the obtained suspension using Miracloth. D. An image of conidiospores on a hemacytometer. Red arrows indicate conidiospores. Scale bars = 200 μ m. E. A plastic tray with a transparent lid containing *Hpa*-inoculated *Arabidopsis* plants.

B. Evaluation of *Hpa* growth by counting conidiospores

1. Harvest aerial parts of *Hpa*-inoculated plants in a 50 ml conical tube with sterile water.

Note: The number of replicates and plants per replicate is dependent on the experimental design. Usually, we harvest 5 pools, each consisting of 3 plants in 1 ml

water (total 15 plants) for each *Hpa*-inoculated *Arabidopsis* line.

2. Vortex the conical tube.
3. Count the number of conidiospores released in water using a hemacytometer (see Figure 1D).
4. Pat the plants dry on paper towels and measure their fresh weight.

Note: Growth of Hpa is represented as the number of conidiospores per ml water per gram fresh weight (conidiospores ml⁻¹ g⁻¹ FW). The intensity of disease symptom (the number of conidiospores) is variable, dependent on several conditions such as humidity, the intensity of light, timing for inoculation and age of host plants. The conditions can be controlled in each experiment by placing all the plant materials (e.g. WT and all the investigating mutants) in the same tray during infection.

C. Evaluation of *Hpa* growth by counting sporangiophores after trypan blue staining

Note: Protective wear should be applied at all steps because trypan blue solution and chloral hydrate are highly toxic chemicals.

1. Harvest aerial parts of *Hpa*-inoculated seedlings in 50 ml conical tube and just cover the harvested seedlings with trypan blue solution.

Note: At least 50 cotyledons for each of the investigating lines should be sampled.

2. Boil for 1 min in water bath in fume hood.
3. Incubate for 1 h at room temperature.
4. Replace trypan blue solution with chloral hydrate solution and leave overnight on Labo shaker at room temperature.
5. Replace chloral hydrate solution with 60% glycerol.
6. Count the number of sporangiophores per cotyledon using a stereomicroscope.

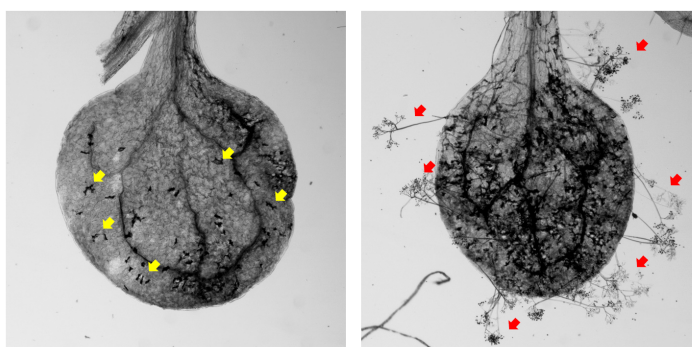


Figure 2. Trypan blue-stained cotyledons. Resistant (left) and susceptible (right) accessions of *Arabidopsis* 7 days after inoculation with *Hpa*. Col-0 plants (left) and Ws-2 *eds1-1* mutants (right) were used as resistant and susceptible accessions of *Arabidopsis* for *Hpa* isolate Emoy2, respectively. Yellow arrows indicate the hypersensitive response (HR), a programmed cell death that is commonly associated with plant disease resistance. Red arrows indicate sporangiophores.

Recipes

1. Trypan blue solution

10 ml lactic acid

10 ml glycerol

10 g phenol

10 ml sterile water

10 mg trypan blue

The working solution is prepared by diluting the above solution with ethanol (1:1 v/v) and storing at room temperature.

2. Chloral hydrate solution

Add around 200 ml of sterile water and 500 g of chloral hydrate to a bottle and stir overnight in a fume hood.

Acknowledgments

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