Chapter 17

Soil-Borne Legacies of Disease in *Arabidopsis thaliana*

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Abstract

The rhizosphere microbiome of plants is essential for plant growth and health. Recent studies have shown that upon infection of leaves with a foliar pathogen, the composition of the root microbiome is altered and enriched with bacteria that in turn can systemically protect the plant against the foliar pathogen. This protective effect is extended to successive populations of plants that are grown on soil that was first conditioned by pathogen-infected plants, a phenomenon that was coined "the soil-borne legacy." Here we provide a detailed protocol for soil-borne legacy experiments with the model plant *Arabidopsis thaliana* after infection with the obligate biotrophic pathogen *Hyaloperonospora arabidopsidis*. This protocol can easily be extended to infection with other pathogens or even infestation with herbivorous insects and can function as a blueprint for soil-borne legacy experiments with crop species.

Key words Plant-microbiome interactions, Rhizosphere, Disease resistance, *Hyaloperonospora arabidopsidis* (Hpa), Biotrophic pathogen

1 Introduction

Plants and their microbial communities are closely intertwined in structure and functioning. By depositing large quantities of their photosynthetically fixed carbon into the soil, plants attract and modulate the composition of the microbial community around their roots [1, 2]. In turn, these micro-organisms influence the plant, for instance by providing it with essential nutrients [3, 4]. The close relationship between plant roots and microbes, coupled with the importance of this so-called rhizosphere microbiome for plant health and performance, has led to the term "plant holobiont," describing the plant not as a single entity but as the sum of the interactions of the individual species [5]. The rhizosphere microbiome can contain both pathogenic and beneficial microbes. Beneficial microbes can control disease by inhibiting the pathogen through antibiosis or competition, but a prominent mode of action is induced systemic resistance (ISR) in the plant.

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ISR entails systemic priming of defense responses, which enables stronger and faster activation of defense responses upon pathogen infection [6, 7]. The development of so-called disease suppressive soils, in which a significant outbreak of disease results in accumulation of microbiota that can control the pathogen, is a prominent example of pathogen control by beneficial microbes [8–11]. Upon encountering stress conditions, such as pathogen attack, plants can change their root exudation profiles [12–14]. The altered rhizodeposition under stress conditions resulting in recruitment of specific beneficial microbes suggested the "cry for help" hypothesis [15]. Recently it was shown that *Arabidopsis thaliana* recruits three strains of beneficial bacteria into its rhizosphere upon foliar infection with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) [16]. The recruited bacteria were isolated in culture and a consortium of a *Xanthomonas,* a *Microbacterium* and a *Stenotrophomonas* sp. was shown to synergistically promote plant growth and induce systemic resistance against *Hpa*. Interestingly, a second generation of Arabidopsis plants grown on soil conditioned with *Hpa*-infected plants showed increased resistance to *Hpa* infection as compared to plants grown on soils conditioned by growing an uninfected first generation of Arabidopsis plants [16]. A similar phenomenon has been observed for *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) infected Arabidopsis plants, where pre-treating the soil with *Pst*-infected plants resulted in reduced disease pressure in a subsequent generation of plants growing on the conditioned soil [12]. The term "soil-borne legacy" was coined in which stress in a first generation of plants results in a microbiome-mediated protection against this stress in a subsequent generation [15]. Here we describe in detail the experimental setup to study the soil-borne legacy in Arabidopsis with *Hpa* as the inducing pathogen.

2 Materials

2.1 Equipment

1. Growth chambers set at 21 °C, 70% relative humidity and 10-h/14-h day/night cycle with a light intensity during the day of 200 μE/m^2/s provided with LuxLine plus F58W/840 cool white tube lamps (Havells Sylvania, London UK).
2. Small autoclave and autoclavable glassware (250 mL Scott bottle).
3. Containers (30–50 L) for mixing soil and water.
4. Dried and sieved soil, preferably a soil that is easy to handle in terms of planting and watering.
5. Round pots (60 mL) with holes in the bottom, to grow plants.

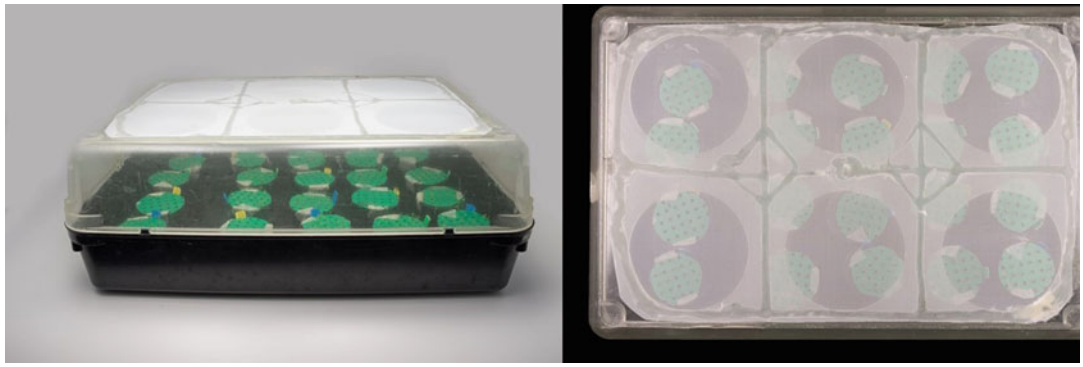

Fig. 1 Trays covered with a gauze lid, to protect the young seedlings, without increasing the relative humidity in the tray. Left side view and right the top view

6. Circular covers of approximately 5 cm in diameter (or to cover the entire pot) are cut from the Greiner bio-one micro-pipette tip 0.5–10 μL holder (Item No: 771280). Make sure approximately 22 holes are included in the soil covers (Figs. 2 and 3).
7. Round petri dishes (6 cm) to serve as dishes for small pots.
8. Trays (approximately 45 × 30 × 8 cm) to contain small pots. Trays should be able to be covered with transparent lids to achieve 100% relative humidity.
9. Transparent lids to cover the trays and transparent lids with large holes covered by gauze (Fig. 1).
10. Carl Zeiss Microscopy, Standard 25 ICS, Item No. 450815.9902 with 100× magnification.
11. Microscope slides.
12. Falcon 15 mL conical centrifuge tubes.
13. Scotch tape.
14. 70% ethanol.
15. 0–1000 μL and 0–2 μL pipettes and corresponding pipette tips.
16. Balance.

2.2 Buffers, Media and Solutions

2.2.1 Arabidopsis thaliana *Cultivation*

1. Seeds of *Arabidopsis thaliana* accession Col-0.
2. 0.2% (w/v) Difco bacteriological agar.
3. Half-strength Hoagland solution [17].
4. Plant labels.
5. Seripettor® bottletop dispenser and bottle to water the pots with the same amount each time.

2.2.2 *Hyaloperonospora Arabidopsidis(Hpa) Noco2 Inoculation*

1. A continuously maintained culture of *Hpa* Noco2 as source of spores. Every week a spore suspension is needed to infect new plants and propagate *Hpa* for further use.

2. Tweezers.
3. Scissors.
4. Falcon 50 mL conical centrifuge tubes.
5. Miracloth (EMD Millipore, Germany).
6. Demi-water.
7. Airbrush; we use a Revell starter kit airbrush connected to the pressurized air of the lab.

2.3 Recipes

2.3.1 Half-Strength Hoagland Supplementation medium (See **Note 1)**

1. 1262 g/5 L (2 mL/L) 5 mM KNO_3
2. 680 g/5 L (2 mL/L) 2 mM KH_2PO_4
3. 1230 g/5 L (2 mL/L) 2 mM $MgSO_4$
4. 28 g/5 L (2 mL/L) 10 μM Fe-EDDHA
5. 2950 g/5 L (2 mL/L) 5 mM $Ca(NO_3)_2$
6. Micronutrients (2 mL/L).
   - 3.90 g/5 L H_3BO_3
   - 9.30 g/5 L KCl
   - 9.30 g/5 L KCl
   - 0.85 g/5 L $MnSO_4 \cdot H_2O$
   - 1.45 g/5 L $ZnSO_4 \cdot 7H_2O$
   - 0.32 g/5 L $CuSO_4 \cdot 5H_2O$
   - 0.22 g/5 L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

3 Methods

The soil-borne legacy experiment consists of two parts. The first part is the conditioning of the soil with either uninfected or *Hpa*-infected Arabidopsis plants. The second part is assessing of the soil-borne legacy effect with an *Hpa*-infected second generation of plants growing on soil conditioned with healthy plants or *Hpa*-infected plants.

3.1 Arabidopsis Cultivation and Infection

1. Suspend the Arabidopsis seeds in sterile 0,2% agar in a 15 mL Falcon tube. To calculate the number of Arabidopsis seeds needed, use: "(number of pots x 30) x 2" to make sure there are plenty of seeds to sow. One hundred Arabidopsis seeds weigh approximately 1.5 mg. Add 10 mL of 0.2% agar per 90 mg of seeds. Imbibe at 4 °C in the dark for 2–4 days (*see* **Note 2**).
2. One day before sowing, mix the soil with the appropriate amount of water or half-strength Hoagland, depending on the type of soil and fill all pots; make sure the pot is filled up to the edge. To assess the soil-borne legacy effect, a minimum of 10 replicates per treatment are needed.

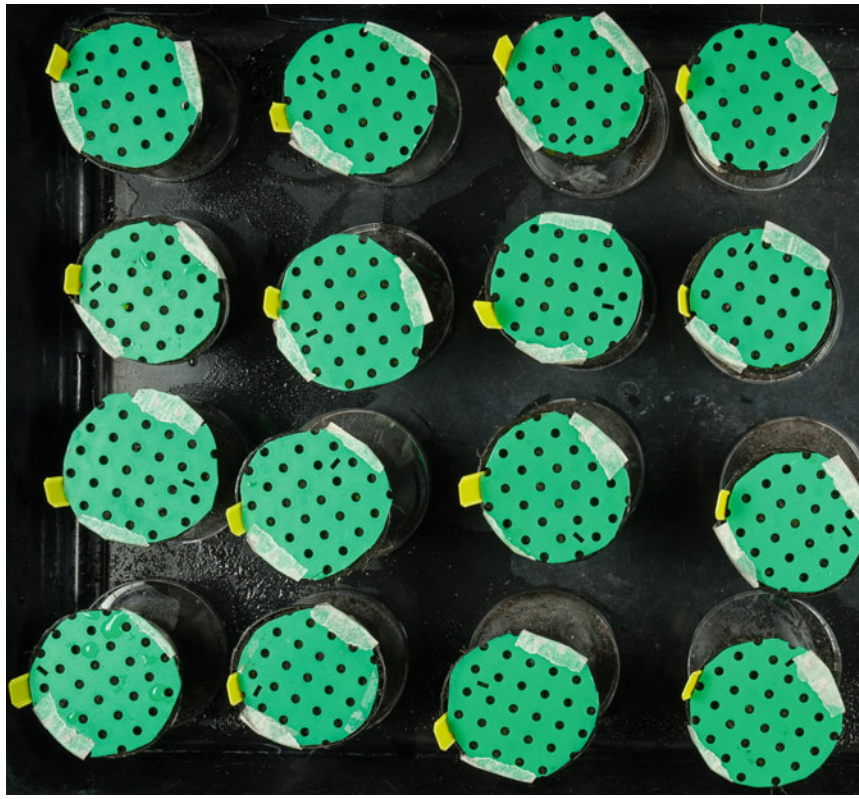

Fig. 2 Top view of 5-cm pots right after sowing Arabidopsis seeds in each of the circular holes of the circular soil covers. The soil covers were cut from a Greiner micro-pipette-tip holder and each placed in a 6-cm Petri dish to allow individual watering and to prevent cross contamination between pots

3. Cover the pots with a soil cover (*see* equipment, Subheading 2.1, **item 6**) and tape it to the side of the pot (Fig. 2). Place each pot in an individual Petri dish bottom or lid to serve as saucers. Store the pots at 4 °C in the dark, until sowing.
4. Sow the seeds on the pot. Using a 0–1000 μL pipette, take up the 0.2% agar with the suspended seeds. Release 1 seed at a time, by turning the volume adjustment of the pipette down slowly. By doing so you are able to control the flow of the pipette better and you are able to so 1–3 seeds per hole in the cover.
5. Randomize the pots (with the Petri dishes) in the trays (Fig. 2). Close the trays with a transparent lid. To make sure the lids are properly closed and to ensure high humidity within the tray, tape the lids to the trays.
6. Move the trays to the plant growth chambers.
7. One week after sowing, change the transparent lids for the lids with gauze to decrease humidity.

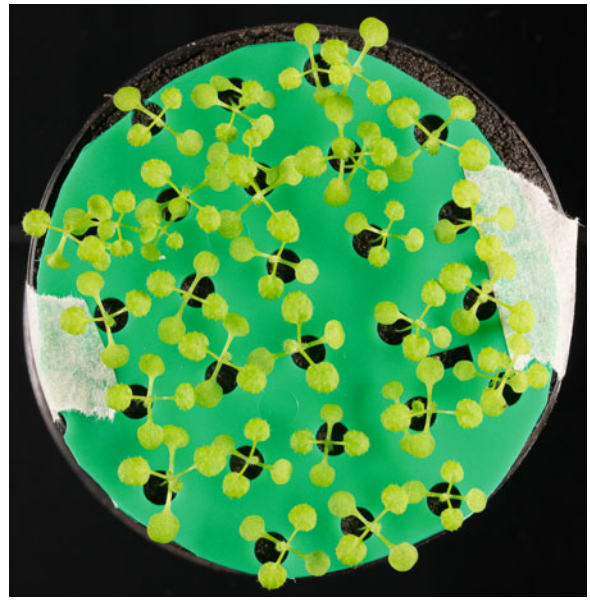

Fig. 3 Two-week-old seedlings right before inoculation with *Hpa*. One to two seedlings per hole have grown and all plants are of similar size. Pots with uneven germination or growth are to be removed from the experiment

8. Water the plants by dispensing 5 mL of water into the Petri dishes in which the individual pots are placed. Add more or less water when needed (*see* **Note 3**).
9. Two weeks after sowing select the plants and pots that are uniform in germination and growth. *See* Fig. 3 for an example of 2-week-old seedlings.
10. Mark the pots and inoculate half of them with an *Hpa* spore suspension according to Asai et al. (2015) [18]. Mock inoculate the other half of the pots with water. In short, collect sporulating seedlings from a maintenance *Hpa*-infected stock of plants and transfer them to a 50 mL Falcon tube. Add approximately 25 mL of sterile water and shake. After filtering through Miracloth, count the number of spores in three separate 1-μL droplets. Adjust the spore density to 50 spores per μL. By using the airbrush, spray the spore suspension over the plants until tiny droplets start to form on the leaves. Let the droplets dry and proceed with the next step. To treat the control group, do exactly the same, except with sterile water instead of spore suspension.
11. Exchange the lids with gauze for the transparent lids without gauze, to increase relative humidity. Tape the lids to the trays and place back in the growth chambers. Cultivate the inoculated and non-inoculated plants for 1 week to allow build-up of the soil-borne legacy.

12. One week after *Hpa* inoculation, cut off all above ground plant parts using a razor blade. For a minimum of 10 replicates per treatment, measure the fresh weight of the plants and count the number of spores. For a detailed description see the spore counting protocol detailed below.
13. Sow a new generation of seeds on the pots, as described in 1 and 4–8. Water when needed, and use half-strength Hoagland solution once during the first week of growing.
14. One week after sowing, exchange the transparent lids for the lids with gauze as in **step 7**.
15. Two weeks after sowing the second generation of seeds, inoculate all replicates with a *Hpa* spore suspension as mentioned in 9–12.
16. One week after inoculation, determine the disease severity by assessing the number of spores per gram of leaf fresh weight as described in the spore counting step described below.

A flowchart of these steps is presented in Fig. 4.

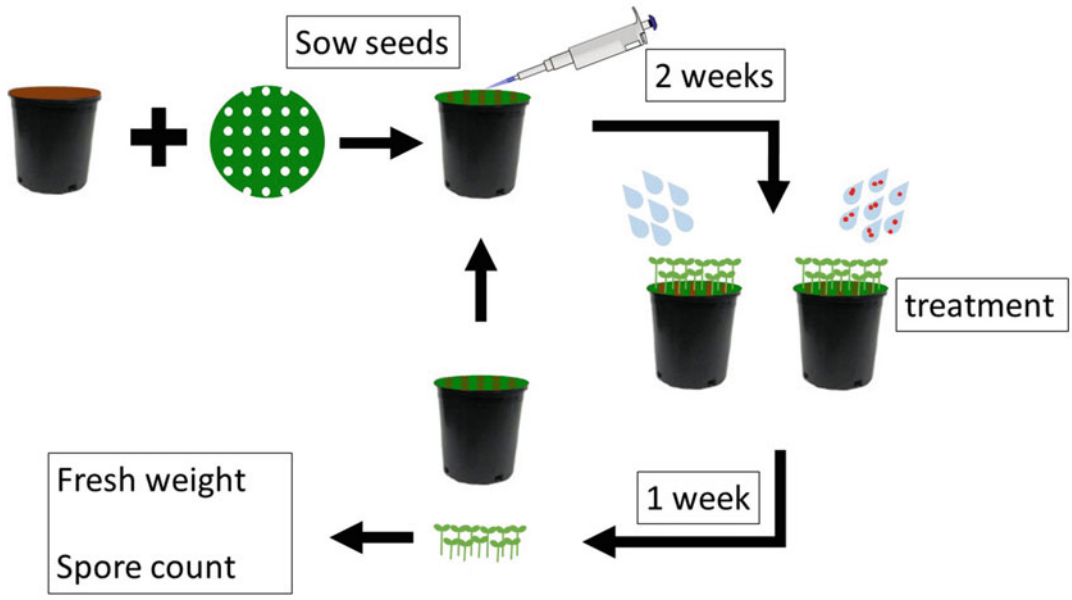

Fig. 4 A flowchart depicting the entire workflow of a soil-borne legacy experiment. (1) Fill the pots with soil and cover the soil with a plastic cutout before sowing the seeds for the first generation of plants. (2) After 2 weeks of growth in the climate chamber, plants are inoculated with either *Hpa* spores or a control solution. During the week that follows the soil microbial community is conditioned by either healthy (uninoculated) plants or by *Hpa*-infected plants. This is the soil-conditioning phase of the experiment. (3) One week after inoculation, the above ground parts of the plants are removed from the pots, and seeds are sown for the second generation of plants. To determine the disease severity in each phase of the experiment, plants are harvested and the number of spores per gram of shoot fresh weight is determined

3.2 Hpa inoculation and Mock Treatment (Step 10)

The method for determining the number of spores per gram leaf fresh weight is derived from Asai et al. [18]. Since *Hpa* is an obligate biotrophic pathogen it can only be propagated on living plant tissue. The Noco2 isolate of *Hpa* can infect Arabidopsis accession Col-0 (*see* **Note 4**).

1. From a maintenance culture of *Hpa* Noco2 infected Arabidopsis Col-0 plants, cut off the leaves that carry *Hpa* sporangia and collect them in a 50 mL Falcon tube.
2. Add 30 mL sterile water to the tube and shake to release the *Hpa* spores from the leaves.
3. Filter the spore suspension through Miracloth.
4. Assess the spore density by counting the number of spores in three separate 1-μL droplets under a microscope.
5. Dilute the spore suspension to 50 spores/μL.
6. Using the airbrush, spray the spore suspension until tiny droplets start to take shape on the leaves.
7. Leave the droplets to air dry.
8. For the mock treatment, spray sterile water on the plants in the same way as the spore suspension.
9. To ensure proper infection, the pots should be placed in trays with closed lids to increase humidity to 100%. Spray the lid on the inside with some water before closing to increase relative humidity inside the tray.

3.3 Spore Counting (Step 12)

The method for determining the number of spores per gram of leaf fresh weight is derived from Asai et al. [18].

1. Pipette 3 mL of sterile water in the 15 mL Falcon tubes.
2. Balance and tare a 15 mL Falcon tube containing 3 mL of water.
3. Cut off all the aboveground plant parts of one pot using a razor blade and collect them in the tared Falcon tube.
4. Weight the tube to determine the fresh weight of the plant material.
5. Shake the tube containing the plant material.
6. Place three 1-μL droplets from the tube containing the plant material on a microscope slide and count the number of spores in each of the droplets under the microscope. Use the average of the droplets to determine the number of spores per μL.
7. Use the fresh weight and the number of spores per μL to determine the spores per gram of leaf fresh weight.

4 Notes

1. We usually prepare 5 L stock solutions and use 2 mL/L of these stocks to make 25 L of half-strength Hoagland at a time. Prolonged storage of the half-strength Hoagland solution will lead to precipitation of some of the salts, so don't make more than you(r) lab will use within 1–2 weeks.
2. The 0.2% agar makes it easy to distribute the seeds with a pipette. It might take some trial and error to get the concentration of seeds right to consistently pipette 2 or 3 seeds per hole without pipetting too much agar. It also ensures proper imbibition of the seeds.
3. All pots are watered by adding water or half-strength Hoagland in the Petri dish in which the individual pots are placed, never from the top of the pot.
4. *Hpa* is an obligate biotrophic pathogen and needs to be propagated on living plants. Each week new plants need to be infected with spores from last week's stock of *Hpa*-infected plants.

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