Chapter 23

Collection of Sterile Root Exudates from Foliar Pathogen-Inoculated Plants

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Abstract

In nature and agriculture, plants interact with an astonishing number of microbes, collectively referred to as the "plant microbiome." Roots are a microbial hotspot where beneficial plant-microbe interactions are established that support plant growth and provide protection against pathogens and insects. Recently, we discovered that in response to foliar pathogen attack, plant roots can recruit specific protective microbes into the rhizosphere. Root exudates play an essential role in the interaction between plant roots and rhizosphere microbiota. In order to study the chemical communication between plant roots and the rhizosphere microbiome, it is essential to study the metabolite profile of root exudates. Here, we describe a detailed protocol for the collection of sterile root exudates that are secreted by *Arabidopsis thaliana* roots in response to inoculation of the leaves with the biotrophic pathogen *Hyaloperonospora arabidopsidis*.

Key words Root exudates, *Hyaloperonospora arabidopsidis* (*Hpa*), Biotrophic pathogen, Hydroponic system, Plant-microbiome interactions

1 Introduction

Plants secrete up to 40% of their photosynthetically fixed carbon into the rhizosphere as root exudates [1]. Root exudates play an essential role in plant-microbe interactions [1, 2]. For decades, scientists investigated different ways to collect plant root exudates. Depending on culture conditions and research purpose, root exudates collection methods have been described for plants grown hydroponically, in sand, or in soil [3, 4]. Previously, we demonstrated that upon foliar pathogen infection, plant roots dynamically shape their root microbiome, resulting in the recruitment of a consortium of microbes that in turn are able to protect the plant against the foliar intruder [5]. In that study, *Arabidopsis thaliana* (hereafter Arabidopsis) plants were inoculated with the oomycete foliar pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), and 7–14 days later, significant changes in the root-associated microbial

Lilia C. Carvalhais and Paul G. Dennis (eds.), *The Plant Microbiome: Methods and Protocols*, Methods in Molecular Biology, vol. 2232, https://doi.org/10.1007/978-1-0716-1040-4_23,

community were detectable. In order to investigate root exudates metabolites that are associated with this microbial recruitment, we needed to develop an axenic system that prevented alteration of the root-excreted compounds by microorganisms. To this end, we developed a root exudates collection system based on a hydroponic plant cultivation system that allowed for microbial inoculations on the leaves, while maintaining a sterile environment for the hydroponically grown roots.

Hpa is the causal agent of downy mildew, which is a disease that affects many leafy crops, and was labelled as one of the top 10 oomycete pathogens in molecular plant pathology [6]. As an obligate biotroph, *Hpa* must be maintained on living plant tissues [7]. This means that when *Hpa* conidiospores are collected for inoculation, also the microbiota from the phyllosphere are co-collected and end up in the inoculum. Hence, during the inoculation of *Hpa*, not only *Hpa* conidiospores are transferred to the plant but also the phyllosphere microbiome that was present on the leaf at the moment of spore collection. The most common way of *Hpa* inoculation is by spraying conidiospores onto the leaves [8, 9]. For studying effects of leaf inoculation on the root exudates metabolite profile, it is essential to avoid contamination of the root system by microbes that are sprayed onto the leaves. To this end, roots must remain effectively separated from the foliar microbiome in a sterile condition. Here, we established a root exudates collection system in which the leaf and root compartments are separated by seed holders. During collection, the whole root exudates collection system was kept sterile in Eco2boxes. Foliar *Hpa* inoculation was conducted by placing small droplets of conidiospores on the leaf surface instead of spraying. Sterile root exudates of *Hpa*-infected *Arabidopsis* plants were successfully collected using this protocol.

2 Materials

Prepare all solutions using demineralized water and analytical grade reagents (unless indicated otherwise). Diligently follow all waste disposal regulations.

2.1 Equipment

1. Clean 1.5-mL Eppendorf tube.
2. Fume hood.
3. A glass desiccator or other device that can be air-tightly closed.
4. 250-mL beaker.
5. Insert: Plastic container (polypropylene (PP), 350 cc, Ø119 mm, transparent, www.paardekooper.nl) (Fig. 1a).
6. Araponic seed holders (www.araponics.com) (Fig. 1b).

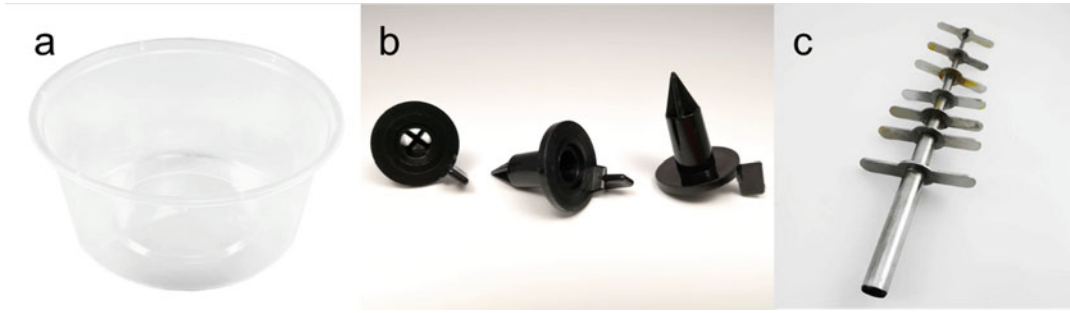

Fig. 1 Pictures of the special equipment used for root exudates collection hydroponic system. (**a**) Plastic container (PP, 350 cc, Ø119 mm, transparent, www.paardekooper.nl) for making the insert. (**b**) Araponic seed holders (www.araponics.com). (**c**) Metal hole punchers of different sizes

7. Metal 1-cm hole puncher (Fig. 1c) (*see* **Note 1**), scissors and tweezers.
8. Eco2box: 1000-cc PP containers with green filter (www.Eco2box.com).
9. 25-mL glass vials.
10. Glass media bottles.
11. Growth chamber set at 21 °C, 70% relative humidity, and 8-h/16-h day/night regime with a light intensity during the day of 200 μE/m^2/s provided by bulb HPI lamps (Philips, Eindhoven, the Netherlands) or LuxLine plus F58W/840 cool white tube lamps (Havells Sylvania, London, UK).
12. Large autoclave (50 L) and autoclavable plastic bags (40 × 60 cm).
13. 9-cm Petri dishes.
14. Autoclavable tape.
15. Microscope (Zeiss Axioskop 2; magnification 10×).
16. P10000, P1000, P200, P2 pipette and pipette tips.
17. Incubator for bacterial growth set at 28 °C, 10-h day/14-h night, Philips TL-D 36 W/33 lamps.
18. Magnet rotors and magnetic stirrer.
19. Miracloth.
20. 50-mL Falcon tubes.
21. Laboratory incubator set at 60 °C.
22. Electronic pipette or Eppendorf repeater pipette.
23. Bunsen burner.
24. Water bath at 100 °C.
25. Tube clip for Eppendorf tube.
26. Water bath floating rack.

27. Fine paintbrush.
28. Glass slides and coverslips.
29. Parafilm.
30. Freeze drying machine.

2.2 Buffers, Media and Solutions

1. Seeds of *Arabidopsis thaliana* (L.) Heynh.
2. Bleach.
3. 37% (w/v) HCl.

2.2.1 Arabidopsis Seed Sterilization and Vernalization

4. Modified Hoagland nutrient solution [10]: 5 mM KNO_3, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 2.5 mM KH_2PO_4, 4.7 mM MES, 70 μM H_3BO_3, 14 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 10 μM NaCl, 0.2 μM Na_2MoO_4, 50 μM Fe(III)-EDTA. Adjust pH to 5.5 with KOH (*see* **Note 2**). Autoclave the prepared Hoagland nutrient solution at 121 °C for 20 min.
5. Half-strength Hoagland agar solution (100 mL): Mix 50 mL modified Hoagland nutrient solution with 50 mL demineralized water to make half-strength Hoagland solution. Add 2 g granulated agar (Difco) and 14.7 g sucrose to the half-strength Hoagland solution to obtain final concentration 0.2% (w/v) and 1.47% (w/v), respectively. Autoclave the prepared half-strength Hoagland agar solution at 121 °C for 20 min.

2.2.2 Hydroponic System

1. Autoclaved tap water.
2. 75% ethanol (v/v).
3. Modified Hoagland nutrient solution: (*see* Subheading 2.2.1, **item 4**).
4. 1/10 Hoagland agar solution (100 mL): Mix 5 mL modified Hoagland nutrient solution with 95 mL demineralized water as 1/10 Hoagland solution. Add 8.5 g granulated agar (Difco) to the 1/10 Hoagland solution to obtain final concentration 0.85% (w/v). Autoclave the prepared 1/10 Hoagland agar solution at 121 °C for 20 min. Keep the TSA medium liquid at around 60 °C in a laboratory incubator.

2.2.3 Sterility Assay with Tryptic Soy Agar (TSA) Medium

1. Tryptic soy agar (TSA) medium (per liter): Add 30 g Tryptic soy broth (TSB) per liter of demineralized water supplemented with 15 g of granulated agar (Difco) for solid medium in Petri dishes (*see* **Note 3**). Autoclave at 121 °C for 20 min.
2. TSA plates: After autoclaving, cool the TSA medium to around 60 °C in a laboratory stove (*see* **Note 4**). Pour approximately 25 mL TSA into each 9-cm Petri dish (*see* **Note 5**). Dry the plates in the laminar flow hood with the lid half open for 30 min. Then seal them with Parafilm, or place them in the plastic bag from which the plates were taken. Store the plates at room temperature (RT) for no more than 2 weeks.

2.2.4 Hpa Infection Assay by Trypan Blue Staining

1. Trypan blue stock solution: 10 mL lactic acid, 10 mL glycerol, 10 g phenol, 10 mL sterile water and 10 mg trypan blue. The working solution is prepared by diluting the stock solution with 96% ethanol (1:1 v/v) and storing at RT [8].
2. Chloral hydrate solution: Add 200 mL of sterile water and 500 g of chloral hydrate to a bottle and stir with a magnet rotor on a magnetic stirrer overnight in a fume hood [8].
3. 60% glycerol.

3 Methods

3.1 Arabidopsis Seed Sterilization and Vernalization

1. Disperse Arabidopsis seeds in a 1.5-mL Eppendorf tube (*see* **Note 6**). Keep the tube cap open and carefully place it horizontally in a desiccator. Place a 250-mL beaker in the desiccator, add 100 mL bleach to the beaker and mix it with 3.2 mL 37% HCl. Quickly close the desiccator and vapor sterilize the seeds for 3–4 h. This step should be conducted in a fume hood because the chlorine gas that is released from the bleach/HCl mixture is toxic.
2. Open the desiccator in the fume hood. Carefully close the tube in the desiccator and transfer the tube with the sterile seeds to a laminar flow cabinet. Keep the tube open in the flow cabinet for at least 30 min to get rid of the chlorine gas. Close the tube. Sterilized seeds can be stored in the dark at RT for 1 month.
3. Add 1 mL half-strength Hoagland agar solution to the Eppendorf tube containing sterilized seeds. Incubate the tube in the dark at 4 °C for 3 days to vernalize.

3.2 Hydroponic System for Sterile Plant Nursery (See Note 7)

1. Cut off the top part of plastic containers (PP, 350 cc, Ø119 mm, transparent) (Fig. 1a) until 4.5 cm deep to make inserts for seed holders. Make 7 holes (equally distributed) on each of the insert (Fig. 2a) with a heated 1-cm metal puncher (Fig. 1c) (*see* **Note 8**).
2. Autoclave the inserts and Eco2boxes (PP, 1000 cc, green filter) separately in autoclave bags at 121 °C for 20 min (*see* **Note 9**).
3. Seal the top (flat) side of the Araponic seed holder with autoclavable tape (Fig. 2b) (*see* **Note 10**). Autoclave the taped seed holders in Eco2boxes at 121 °C for 20 min.
4. Work in a laminar flow cabinet. Sterilize tweezers by dipping them in 75% ethanol then quickly burn the alcohol from the tweezers with a Bunsen burner.
5. Work in a laminar flow cabinet. Pick up a taped seed holder with sterile tweezers (Fig. 2c), place it upside down on a sterile Petri dish (Fig. 2d). Fill up the seed holder slowly from the

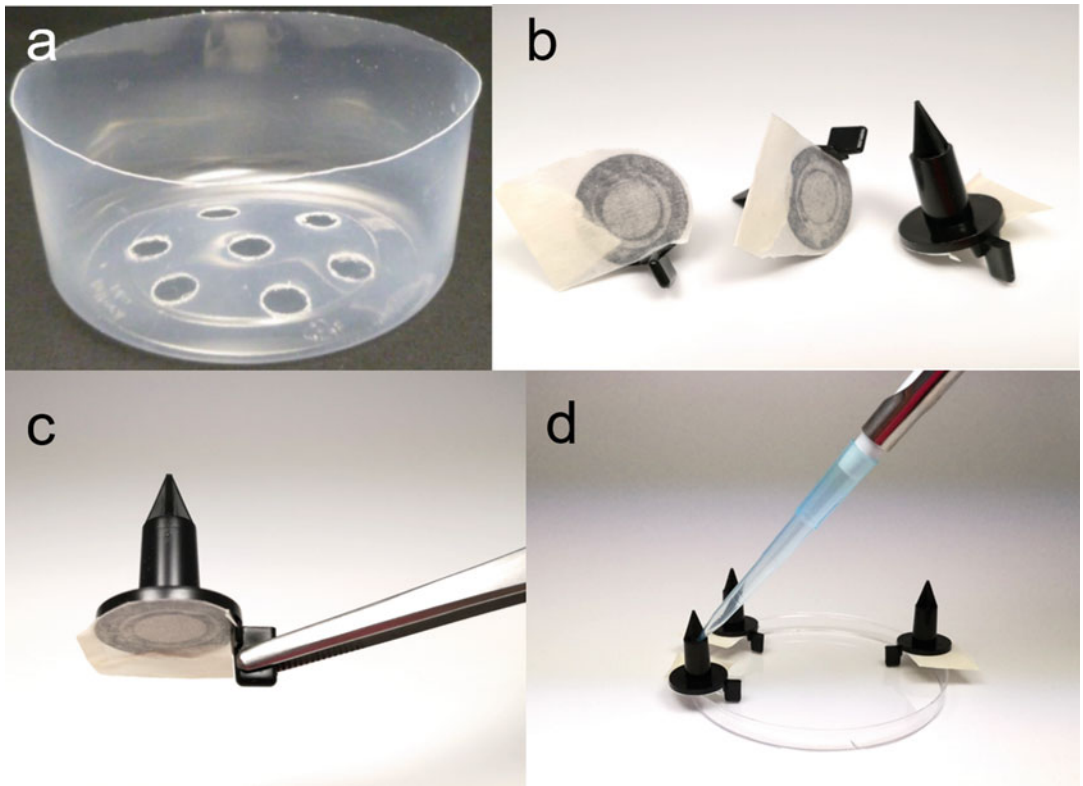

Fig. 2 Preparation for the insert and seed holders. (**a**) The prepared insert with 7 equally distributed holes. (**b**) Araponic seed holders sealed with autoclavable tape. (**c**) An example of how to pick up a seed holder with tweezers. (**d**) Place taped seed holders upside down on a sterile Petri dish and fill them up from the pointed bottom slowly with 1/10 Hoagland agar solution

pointed bottom with 1/10th strength Hoagland containing 0.85% agar (Fig. 2d). Avoid forming any air bubbles during this step (*see* **Note 11**).

6. Wait for the agar in the seed holder to solidify and remove the tape with sterile tweezers. Prepare all the seed holders according to Subheading 3.2, **steps 5** and **6**.
7. Work in a laminar flow cabinet. Sterilize the tweezers as described in Subheading 3.2, **step 4**. Open the autoclave bags with autoclaved inserts and Eco2boxes inside the laminar flow cabinet. Then assemble them with the cooled down sterilized tweezers as in Fig. 3a.
8. Work in a laminar flow cabinet. Fill Eco2box with 200 mL sterile modified Hoagland nutrient solution. Keep the bottom of the insert approximately 1 cm above the modified Hoagland nutrient solution (*see* **Note 12**; Fig. 3b). Carefully put an agar-filled seed holder in each of holes of the insert with sterilized tweezers. Make sure there are no air bubbles attached at the bottom of the seed holders (Important!) (*see* **Note 13**).

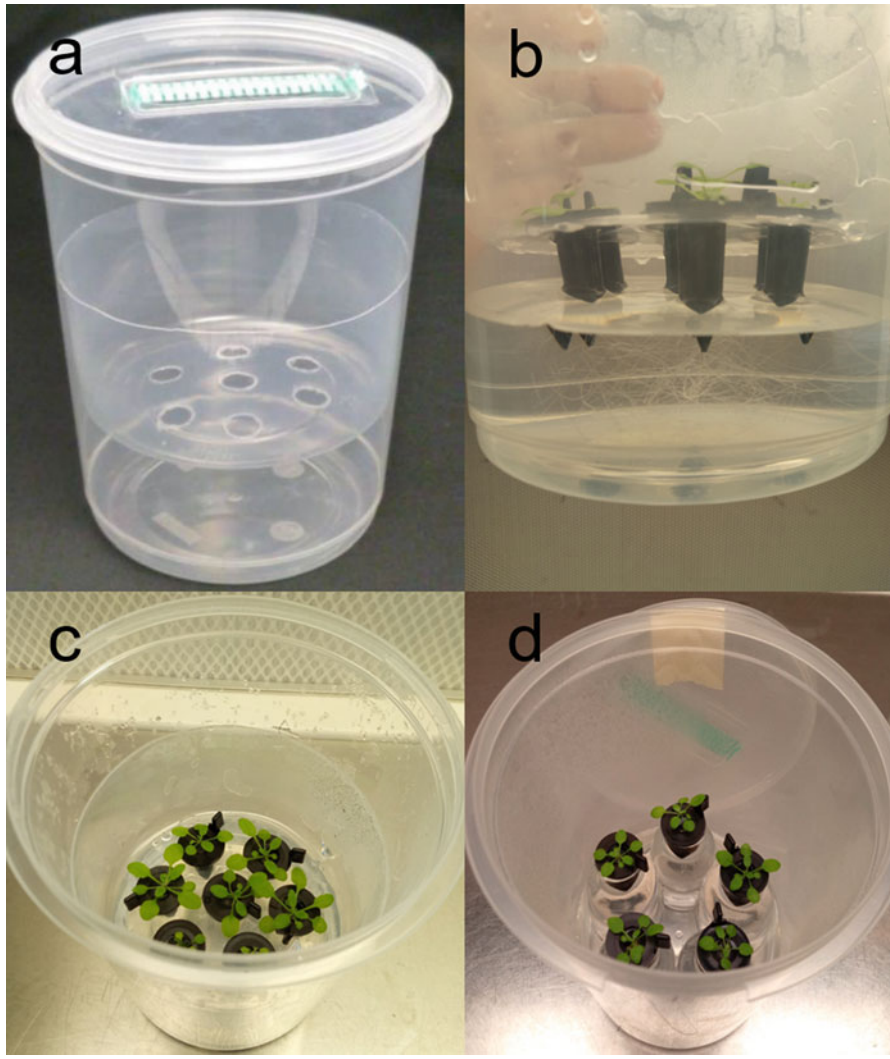

Fig. 3 Hydroponic system setup. (**a**) The assembly of the insert and Eco2boxe. (**b**) Keep the bottom of the insert approximately 1 cm above the modified Hoagland nutrient solution. (**c**) Two-week-old Arabidopsis seedlings growing in the hydroponic system for sterile plants nursery. (**d**) Arabidopsis seedlings transferred from the plant nursery to the root exudates collection hydroponic system

9. Work in a laminar flow cabinet. Resuspend the vernalized seeds with half-strength Hoagland agar solution until the seeds are equally distributed in the solution (*see* **Note 14**). Sow 2–3 seeds on the agar in the middle of each seed holder with a P20 pipette. Gently close the lids of the Eco2boxes after sowing on all seven seed holders in the box.
10. Put the prepared hydroponic system in a long-day plant growth chamber (21 °C, 70% humidity, 16-h light/8-h dark) for 2 weeks (Fig. 3b and c).

11. Check at 5 days after sowing, if more than one seed germinated on one seed holder, gently remove all seedlings except for the largest one with sterile tweezers in a laminar flow cabinet.

3.3 Hydroponic System for Root Exudates Collection

1. Label 25-mL glass vials with numbers (*see* **Note 15**). Autoclave Eco2boxes and glass vials in autoclave bags.
2. Work in a laminar flow cabinet. Place 5 glass vials in the Eco2-box. Fill the glass vials with modified Hoagland nutrient solution using a pipette until it overflows (*see* **Note 16**). Add approximately 80 mL sterile water to the bottom of the Eco2-box to keep the relative humidity (RH) high inside the Eco2-box (*see* **Note 17**).
3. After 2 weeks of growing in the nursery system (Fig. 3b, c), select seedlings of uniform size and carefully transfer them together with the seed holders to sterile glass vials containing fresh Hoagland solution for root exudates collection (Fig. 3d). Root exudates of plants infected by *Hpa* should be collected by collecting the sterile Hoagland nutrient solution in which the *Hpa*-infected plants are grown for 5 days (*see* **Note 18**).

3.4 Foliar Hpa Inoculation

1. Take a plastic tray with a transparent lid containing Arabidopsis plants that are densely covered by *Hpa* sporangiophores. For this, a *Hpa* strain that is virulent on the Arabidopsis accession used is required. Here, we used *Hpa* strain Noco2, which is virulent on Arabidopsis accession Col-0 [11]. Open it in a fume hood to avoid dispersal of conidiospores (*see* **Note 19**).
2. Harvest *Hpa* Noco2 conidiospores from infected Arabidopsis seedlings, as described by Asai et al. [8, 12] with minor modifications. Briefly, avoiding any soil contamination, harvest aerial parts of the *Hpa*-infected seedlings with sterile scissors and tweezers into a 50-mL Falcon tube until it is half full. Add approximately 15 mL of autoclaved tap water into the 50-mL Falcon tube and wash off the sporangia by gentle shaking. Filter the obtained conidiospores suspension using Miracloth. Collect the filtrate containing the *Hpa* sporangia in a new 50-mL Falcon tube.
3. Shake the tube with the *Hpa* spore suspension to mix. Place three 1-μL droplets of *Hpa* spore suspension on a glass slide and count the number of conidiospores under microscope. Adjust the density of the conidiospore suspension to 50–100 spores/μL. Use this as the inoculum.
4. Work in a laminar flow cabinet. Use an Eppendorf repeater pipette, apply 3–4 1-μL drops of the inoculum on the surface of every leaf (Fig. 4a). Re-mix the inoculum by vortexing gently and briefly after the inoculation of each plant. For the control plants, apply 3–4 1-μL drops of autoclaved tap water on each leaf.

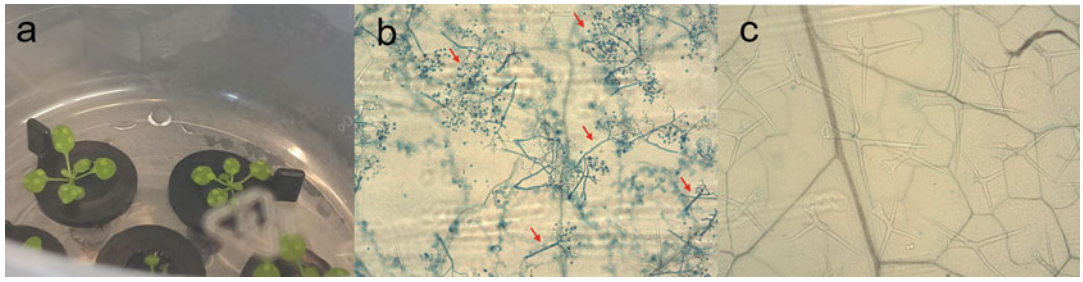

Fig. 4 *Hpa* inoculation and infection assay by trypan blue staining. (**a**) Arabidopsis seedlings inoculated with 1-μL drops of *Hpa* inoculum on the leaf surface. (**b**) Trypan blue-stained, *Hpa*-infected leaf of Arabidopsis seedling, 5 days after *Hpa* inoculation. Red arrows indicate sporangiophores carrying the *Hpa* conidia (spores). (**c**) Trypan blue-stained healthy leaf of Arabidopsis seedling of control treatment

5. Gently close the Eco2boxes and carefully transfer the hydroponic systems for root exudates collection back to the long-day growth chamber (21 °C, 70% humidity, 16-h light/8-h dark) (*see* **Note 20**).

3.5 Root Exudates Collection

1. Work in a laminar flow cabinet. Five days after *Hpa* inoculation, harvest the leaves of both *Hpa*-inoculated and control plants by cutting at the root shoot junction using small scissors. Store all the harvested leaves of each plant in one 1.5-mL Eppendorf tube (*see* **Notes 21** and **22**). Label the Eppendorf tubes according to the number on the glass vials (*see* **Note 15**).
2. Work in a laminar flow cabinet. Transfer the collected root exudates from the glass vials to 50-mL Falcon tubes using a P10000 pipette. Label the Falcon tubes according to the number on the glass vials (*see* **Note 15**).
3. Work in a laminar flow cabinet. Pipette 10 μL of the collected root exudates from each Falcon tube onto a TSA plate. Briefly dry the plates, then seal with Parafilm. Incubate at 28 °C for 36 h to check whether the root exudates were sterile.
4. Store collected root exudates samples from healthy and *Hpa*-inoculated plants at −80 °C.
5. Stain the harvested leaves with trypan blue solution to observe *Hpa* infection as described by Asai et al. [8, 12] with minor modifications. Briefly, add trypan blue working solution to the 1.5-mL Eppendorf tube until the leaf samples are covered.
6. Use Eppendorf tube clip to close the 1.5-mL Eppendorf tubes with leaf samples (*see* **Note 23**). Put the 1.5-mL Eppendorf tubes on a floating rack. Incubate them for 1 min in water bath at 100 °C in a fume hood.
7. Incubate the boiled samples for 1 h at RT.
8. Replace the trypan blue solution with chloral hydrate solution and incubate overnight at RT in a fume hood.

9. Replace chloral hydrate solution with 60% glycerol. Pick up the leaves gently with tweezers and place them on a glass slide. Spread the leaf carefully with a fine paintbrush without breaking the leaf. Add some 60% glycerol onto the slide if the leaf is too dry to spread. Cover the slide with a coverslip.
10. Use microscope (Zeiss Axioskop 2; magnification 10x) to assess the disease severity of the infected leaves and take pictures (Fig. 4b, c).
11. Discard contaminated root exudates samples according to sterility assay. Select root exudates samples of *Hpa*-infected plants according to *Hpa* infection assay.
12. Freeze dry collected root exudates samples into powder. Store at −80 °C for downstream analysis.

4 Notes

1. The diameter of the hole puncher should be around 1 mm bigger than the diameter of the seed holder.
2. Make 20× concentrated stock solutions and store them in the dark at RT. Prepare a Hoagland nutrient working solution by filling a 1-L solution bottle with 0.5 L demineralized water (Important: do not mix undiluted stock solutions!); after that, add 50 mL of each of the stock solutions and mix well before adding a new stock solution; fill the bottle up to 1 L with demineralized water and mix the working solution well.
3. To make 1 L of TSA medium, place a 2-L Erlenmeyer flask containing 650 mL demineralized water and a magnet on a magnetic stirrer. Add 30 g of TSB and wait until it is mixed completely. Adjust with demineralized water to obtain 1 L, mix and pour 500 mL into two 1-L bottles. Add 7.5 g agar to each bottle and autoclave.
4. Agar starts to solidify at about 50 °C. Alternatively, you could cool the bottle of TSA medium in air, but inevitably you would forget about it and come back finding that solidification had already started. In this case, you can warm the medium up using microwave and cool it down to approximately 60 °C again.
5. If there are any bubbles in the plates, puncture them with a sterile pipette tip while the medium is still liquid and warm.
6. The number of seeds for sterilization should be approximately 3 times more than the number of plants needed in the experiment. One-hundred Arabidopsis seeds weigh approximately 1.5 mg [13].

7. This hydroponic system for sterile plant nursery step is optional. You can grow sterile plants directly in the hydroponic system for root exudates collection (*see* Subheading 3.3). In our experiment, we found that for seeds directly sowed in the hydroponic system for root exudates collection, around 25% of the seedlings die after 1 week, as the agar dried out before the roots could penetrate it and reach the Hoagland solution. For this reason, we added the plant nursery step (*see* Subheading 3.2). In this nursery step, the humidity is higher because of the larger evaporation surface and plant survival rate can reach 100%.
8. You can make this step more efficient by stacking up to 5 inserts together then make each hole all the way through the 5 inserts at once with a heated puncher, instead of doing it one by one.
9. Stacking the inserts and Eco2boxes separately can save a lot of space in the autoclave instead of assembling them first.
10. Seal the seed holder well to avoid leakage. Tape the sticky side of the autoclavable tape together at the edge of the seed holder to form a handle (Fig. 2b), which can be easily grabbed by tweezers to remove the tape in Subheading 3.2, **step 6.** The number of prepared seed holders should be at least twice the number of seedlings needed in your experiment.
11. After solidification, air bubbles form a barrier for water flow and root penetration. To avoid this, make sure there are no bubbles in the pipette tip before filling up the seed holders, pipette the 1/10 Hoagland agar solution slowly and always leave a small volume in the tip instead of dispensing the volume completely.
12. Around 1-cm distance will ensure that the bottom of the seed holders touches the Hoagland solution, while the solution won't spill over the insert and make contact with the (contaminated) leaves.
13. Air bubbles form a barrier for water-uptake from Hoagland solution to the agar in the seed holder. After putting the seed holders into the insert, check from the bottom of the Eco2-boxes if there are air bubbles attached to the tip of the seed holder. If yes, pick the seed holder up and drop down several times until the bubbles disappear.
14. The 0.1% agar prevents the seeds from sinking to the tube bottom and allows an equal distribution of the seeds in the solution. Try with a P20 pipette, if it is difficult to pipette seeds out one by one, add more half-strength Hoagland agar solution to dilute the seeds.

15. This number would be the label of the plant, as well as the label of corresponding root exudates and leaves sample from this plant. This will help you to keep on track in the sterility assay and *Hpa* infection assay (*see* Subheading 3.5, **steps 1–11**).
16. When transplanting from the sterile plants nursery system to the root exudates collection system, the roots of the seedlings are around 3-cm long and it is difficult to place root in a 25-mL glass vial (Fig. 3b). Fill the glass vials until they overflow a bit can prevent the roots from clinging to the side of the vial significantly and make transplanting way easier.
17. High RH (90–100%) is required for *Hpa* infection [8]. Besides, it can prevent the agar in the seed holders from drying out.
18. Normally, root exudates are collected in water for 12–48 h. As it takes more than 2 days for the development of *Hpa* infection and collecting in water for a longer period would cause plant nutrients deficiency, we suggest collecting root exudates in sterile modified Hoagland nutrient solution for 5 days.
19. As *Hpa* is an obligate biotrophic pathogen, it can only survive on living plants. Usually, we take the Arabidopsis seedlings after 1 week of infection for conidiospores collection.
20. Take care with this step. Any sudden movements could shake off the droplets of the *Hpa* inoculum on the leaves and reduce infection. One option is to dry these droplets in the flow cabinet before we close the lids then move the root exudates collection system back to the growth chamber, but this will cause a clear decrease of *Hpa* infection.
21. If the leaf samples would be carried on for the trypan blue staining assay on the same day, they can be stored at RT. Otherwise, the samples can be stored at −20 °C, but this is not recommended.
22. During the trypan blue staining assay for *Hpa* infection, we found that not all the inoculated leaves (leaves formed during the 5 days of root exudates collection are not inoculated) had been successfully infected by *Hpa*. This means if we randomly choose 1–2 leaves from each seedling for the *Hpa* infection assay, we might wrongly assume the plant is not infected and discard the root exudates samples of the plants that are actually successfully infected. To this end, we decided to harvest and stain all leaves of each seedling. If there are more than 2 leaves of one seedling showing clear sporangiophore structures and hyphae, we considered this seedling infected by *Hpa*.
23. The tube clip is recommended when Eppendorf 1.5-mL tubes are heated above 80 °C. Without the clips, tubes would pop open during boiling.

Acknowledgments

The authors would like to thank Diana van Houten for her contribution during designing and testing the hydroponic system and members of the Plant-Microbe Interactions group who had developed the preceding protocols. Annemiek Andel, Joyce Elberse, and other members of the team of Guido Van den Ackerveken are acknowledged for maintenance of *Hpa* cultures. This work is supported in part by the scholarship from China Scholarship Council (CSC).

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