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An Easy and Flexible Inoculation Method for Accurately Assessing Powdery Mildew-Infection Phenotypes of Arabidopsis and Other Plants

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Abstract

Reducing crop losses due to fungal diseases requires improved understanding of the mechanisms governing plant immunity and fungal pathogenesis, which in turn requires accurate determination of disease phenotypes of plants upon infection with a particular fungal pathogen. However, accurate disease phenotyping with unculturable biotrophic fungal pathogens such as powdery mildew is not easy to achieve and can be a rate-limiting step of a research project. Here, we have developed a safe, efficient, and easy-to-operate disease phenotyping system using the Arabidopsis-powdery mildew interaction as an example. This system mainly consists of three components: (i) a wooden inoculation box fitted with a removable lid mounted with a stainless steel or nylon mesh of ~50 µm pores for inoculating a flat of plants with fungal spores, (ii) a transparent plastic chamber with a small front opening for minimizing spore escape while conducting inoculation inside, and (iii) a spore-dislodging and distribution method for even and effective inoculation. The protocols described here include the steps and parameters for making the inoculation box and the plastic chamber at a low cost, and a video demonstration of how to use the system to enable even inoculation with powdery mildew spores, thereby improving accuracy and reproducibility of disease phenotyping.

Introduction

Powdery mildew is one of the most common and important diseases of numerous food crops and ornamental plants[1]. Studies of powdery mildew diseases have been very popular, as evidenced by over 10,500 publications as the search result with "powdery mildew" as key word at the Web of Science (as of November 2020). Indeed, powdery mildew (represented by *Blumeria graminis*) is considered to be one of the top 10 fungal pathogens by the journal

of *Molecular Plant Pathology*[2]. Quantification of disease susceptibility is a necessary step in characterization of plant genes contributing to disease resistance or susceptibility, or functional identification of candidate effector genes in powdery mildew. However, reliable disease phenotyping is far more challenging with powdery mildew compared to that with most other fungal pathogens, partly because, unlike spores of the latter, spores of powdery mildew species (such as *Golovinomyces cichoracearum* UCSC1 based on our lab experience) show reduced viability after going through a water-suspension process[3,4]. Inadequate and/or uneven inoculation of test plants with a particular powdery mildew pathogen may lead to inaccurate phenotyping results.

A number of inoculation methods were reported for powdery mildew studies. These include (i) brushing spores directly from infected leaves to test plants[5], (ii) spraying a spore suspension to test plants[6], (iii) blowing spores using a vacuum-operated settling tower to plants at the bottom of the tower[7], and (iv) spore delivery by the combinatorial use of a nylon mesh membrane and sound-based vibration[8]. The spore-brushing (or dusting) method is easy to perform but uneven in nature, thus it may not be accurate for quantitative assessment. Spore-spraying is convenient and even, but as stated above may result in poor spore germination[4]. The latter two (i.e., iii-iv) are much-improved methods capable of achieving even inoculation; however, both are not flexible in adjusting their inoculation capacity in terms of the number of plants to be inoculated in a single event, making either apparatus is not trivial, and their operation is restricted to lab areas where there is a vacuum and/or electricity source.

Our lab has been working with plant-powdery mildew interaction for over 20 years[9,10]. Over the past decade, we tested a number of inoculation methods and recently developed a simple and yet effective powdery mildew inoculation method. This mesh-based spore-brushing method can ensure even inoculation, and is simple and scalable, thus should be easily adopted by any laboratory working with powdery mildew.

Protocol

1. Making a standard inoculation box with a removable top lid mounted with a mesh

1. Purchase a roll of 50 µm nylon membrane mesh or 48 µm stainless steel mesh (recommended) from stores. Make sure to order enough for cutting into multiple pieces of 14 in x 26 in for replacement of worn mesh.
2. Purchase one 1/4 in x 2 ft x 4 ft medium density fiberboard or plywood, and cut two 24-1/2 in x 10 in pieces and two 12 in x 10 in pieces for making an inoculation box.Use 8 corner clamps for supporting the four sides.
   NOTE: If facing the broad side of the enclosure, the left 12 in x 10 in side is attached permanently and the right 12 in x 10 in side is a removable door held in place by four magnetic catches (**Figure 1A,B**).
3. Place four 2-in corner braces two in each inside corner of the left side, an inch from the top and an inch from the bottom, mark and drill a hole. Use screws to permanently attach the left short side to two long sides (front and back). The inner distance between the two long sides should be 12 in and not 11-1/2 in.
4. With the corner clamps still in place and proper alignment of the right door, mark and drill the location of the 4 magnetic catches to hold the right detachable door in place. Install a button knob to the door. The top of the door catches under the top lid (**Figure 1A,B**).

5. To make a removable top lid with a mesh, cut the long wooden square dowel into 2 sets of 26 in and 12-3/4 in. Glue and nail the four pieces in such a way that it will fit around the box - the inside dimensions of the frame being a little bigger than the outside dimensions of the box, as the screen mesh will be attached to this frame.
6. Cut the screen mesh to a little less than 14 in x 26 in. Do not allow the screen to protrude past the frame so as to prevent sharp edges. Have assistance to stretch the screen on the frame. Hold the mesh with the frame by clamps (**Figure 1C**) and use a stapler gun with the right staples to secure the screen to the frame.
7. Cut two sets of shorter wooden square dowel (21 in and 9 in), and use screws to tightly sandwich the mesh along the frame using the 4 shorter pieces of wooden square dowel (**Figure 1C, D**).

2. Making an inoculation chamber

1. Purchase clear acrylic sheets and hardware items from appropriate stores. Cut a 7 in x 20 in opening window on the front side acrylic sheet of the box as shown in **Figure 2C**.
2. Assemble the left and right sides, front (with window) and rear acrylic sheets into a box using 8 corner clamps as shown in **Figure 2A**. Ensure that the inner measurements of the chamber are equal to the dimensions of the top sheet.
   NOTE: The right side is there for support and will not be attached to the front or rear sheets. It is ½ in shorter than the other sheets on purpose. This right side will become a "draw down" "flap" door held in place by a make-shift hinge at the bottom and two magnetic catches at the top as shown in **Figure 2C,D**.
3. Use corner braces to secure the sheets together (**Figure 2B,C**). Hold the braces in place, mark with a sharpie marker and then drill the hole.
   NOTE: Drilling acrylic is different from drilling anything else, do not force or the side of the hole opposite the drill may crack. Use rivets or screws. Do not overtighten the screws or the acrylic may crack with time.
4. Turn the enclosure on its rear side and remove the 4 corner clamps and position the top sheet in place. Mark and drill.
5. Install the make-shift hinge, made out of a ¾ in angle brace. Use a nylon retaining lock nut as a hinge rod. Do not tighten, leave a gap so the door will rotate on this hinge.
6. Mark, drill and attach the magnetic door catch using screws.

3. Inoculating plants in flats

1. Prepare fresh powdery mildew spores as inocula (e.g., *Arabidopsis* powdery mildew *Golovinomyces cichoracearum* UCSC1). Grow clean *Arabidopsis* immune-compromised *pad4-1* mutant plants [11] in a growth chamber (22 °C, 65% relative humidity for 6-8 weeks), and inoculate one or half flat of *pad4-1* plants to build up sufficient fresh powdery mildew spores (**Figure 3A**).
2. Grow the plants for determining infection phenotypes in standard 11 in x 21 in x 2.5 in flats.
   NOTE: *Arabidopsis* plants grown under short-day (8 h light, 16 h dark; 22 °C) for six to eight weeks are good for infection tests with powdery mildew.
3. When fresh powdery mildew spores from infected plants at 8-10 days post-inoculation (dpi) are available, move

a flat of plants for infection test into the inoculation box inside the inoculation chamber (**Figure 1B,2C**).

4. Cut 15-20 heavily infected *Arabidopsis* leaves (or 4-6 infected rosettes, depending on the leaf/rosette size and level of inoculation), let the leaves dry if there is condensed water.

5. Grab 1-2 leaves or a rosette with a pair of long forceps using one hand and face the leaves upside down. Then, put both hands through the opening of the chamber and gently hit the arm of the forceps with a pair of scissors while slowly moving the leaves/rosette above the mesh mounted on the inoculation box (**Figure 3B**). Repeat this until all the leaves or rosettes are used.
   NOTE: Mature spores will dislodge easily from conidiophores of the infected leaves. Try to dislodge spores on the mesh as evenly as possible.

6. Use one or two fine fan-blender brushes to gently brush the spores through the mesh (**Figure 3C**). Ensure to brush the entire surface of the mesh in different directions for four or more times to maximize even distribution of spores onto the plants in the bottom of the inoculation box (**Figure 3D**). Gently pat the mesh for a few times with the brushes to shake off the spores that are stuck in the mesh.

7. Let the spores settle down for half a minute. Then, move out the flat containing the inoculated plants from the inoculation box. Cover the flat with a plastic dome and place it in a growth chamber (22 °C, 65% relative humidity). Disease phenotyping can be performed at 5, or 8 to 12 dpi [12].

4. Inoculate plants with smaller inoculation boxes

NOTE: In cases where fewer plants are to be inoculated, the standard inoculation box can still be used. Make sure to place the plants in the middle of the box. Dislodge and brush spores in the area of the mesh that covers the plants to ensure all plants are inoculated while saving inocula. Alternatively, and preferably, smaller inoculation boxes can be used (as described below).

1. Convert cardboard boxes to an inoculation box. Simply remove the top and bottom sides of the box, tape the corner to firm it if necessary, and mount a suitably sized 50 µm mesh on the top by gluing or stapling (**Figure 4A,B**).

2. Move the flat containing the plants inside the inoculation chamber, place the smaller inoculation box on top of the flat to cover the plants.

3. Dislodge and brush spores as described above.

5. Inoculate detached leaves in Petri dishes

NOTE: In cases where (i) fresh powdery mildew spores are very limited and/or (ii) plants must be kept clean while disease phenotypes and/or protein subcellular localization in infected cells need to be assessed, detached leaves can be used for infection in MS-agar plates.

1. Make a mini inoculation box that is only slightly bigger than a Petri dish.

2. Prepare ½-strength Murashige and Skoog (MS) medium agar (1.5%) plates. Add thiabendazole (40 mg/L) to reduce mold contamination if required. Store the plates at 4 °C in a fridge until use.

3. Cut one or two fully expanded leaves from the base of the petiole for each individual plant to be tested. Insert the petiole of the detached leaves into agar medium at an angle of ~30°.

   NOTE: Each plate can contain 20-40 leaves depending on the size of the leaves and the plate used (**Figure 4C**).

4. Inoculate the leaves inside the inoculation chamber as described above. Move the plate with the lid to a growth chamber.

5. Cut a small section of a leaf and check the subcellular localization of proteins at specific time-points.

6. For assessing infection phenotypes, transfer the leaves to a fresh aseptic MS-agar plate at 4 or 5 dpi. Cut the base of the petiole before inserting the leaves into the fresh agar medium. Assess the disease phenotypes at 8 or 9 dpi.

Representative Results

Here, we present a new powdery mildew spore inoculation method that is easy to prepare, operate and adjust. **Figure 1** shows the assembly of the standard inoculation box with emphasis on the make of the removable lid mounted with a 50 µm membrane mesh. **Figure 2** shows the assembly of the inoculation chamber. **Figure 3** illustrates the key steps of the inoculation process using this system. **Figure 4** shows other inoculation boxes that can be used to inoculate a whole flat or fewer plants, or detached leaves on MS-agar medium. Finally, **Figure 5** provides data to demonstrate the inoculation evenness as reflected by spore distribution or plant infection phenotypes.

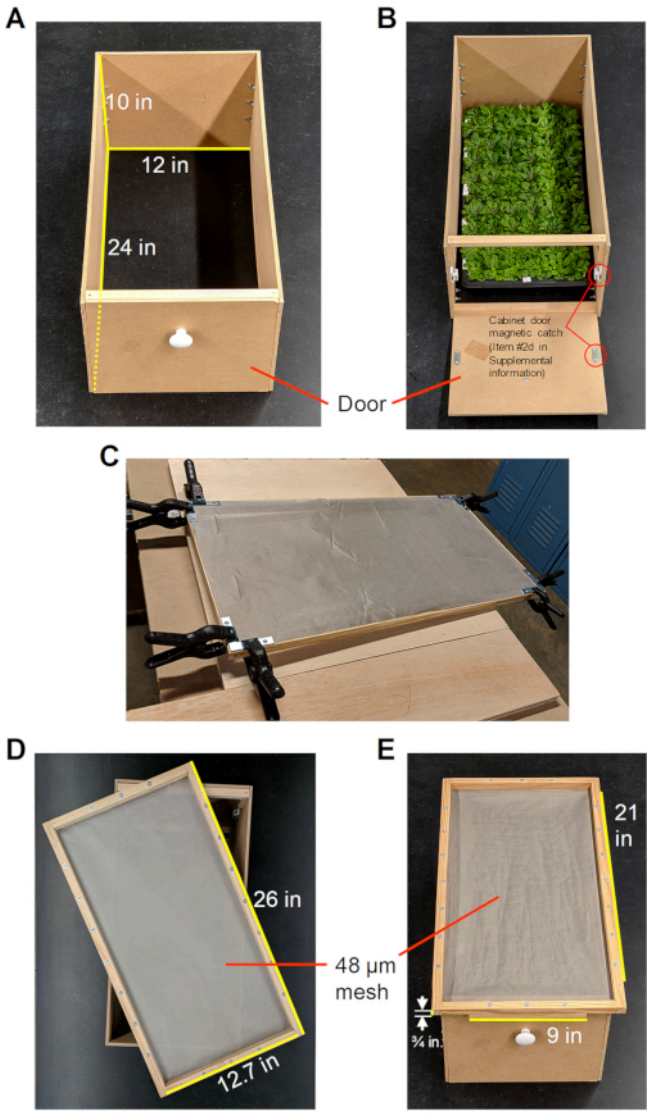

Figure 1. Making an inoculation box. (A-B) Photos showing the box with the door closed (**A**) or open for inserting a flat of *Arabidopsis* plants (**B**). Note a pair of cabinet door magnetic catches are used to hold door. **(C)** A photo showing the mounting of a mesh to the rectangle frame as a critical step for making the lid of the box. Note clamps and corner brackets are used to position the mesh before it is fixed by screwed nails. **(D-E)** Photos showing the removable lid of the box with a new stainless-steel mesh mounted (**D**) or an assembled box (**E**). Note the mesh is fixed between 3/4 in. wood square dowel with indicated length in **D** and **E**. Yellow lines highlight the size measurement of the box. Having an inoculation box that fits a standard flat can greatly enhance phenotyping efficiency especially when a lot of plants are to be inoculated (e.g., during a genetic screen). A removable lid mounted with a mesh makes cleaning or replacement of the mesh easier. Please click here to view a larger version of this figure.

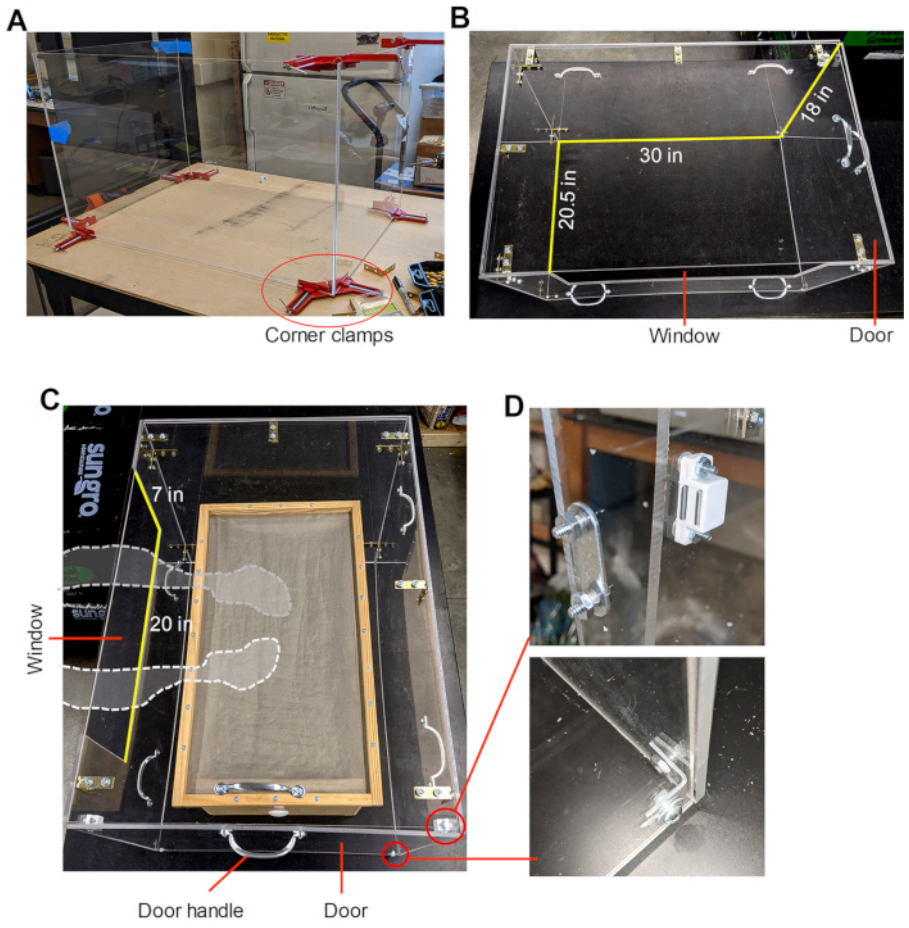

Figure 2. Making an inoculation chamber. (A) A photo showing the initial assembly of the four acrylic sheets using corner clamps. **(B)** A photo giving a top-down view of the plastic chamber. **(C-D)** Photos showing the fully assembled inoculation system (**C**), and one of the two magnetic catches (upper) and make-shift hinges (bottom) installed to one side acrylic sheet as the door (**D**). Note the inoculation window is for dislodging and brushing spores by the user (depicted by dished lines). The yellow lines highlight the size measurement of the box. Having an inoculation chamber is a plus, but without it, inoculation can still be performed with an inoculation box in a small wind-still room or environment. Please click here to view a larger version of this figure.

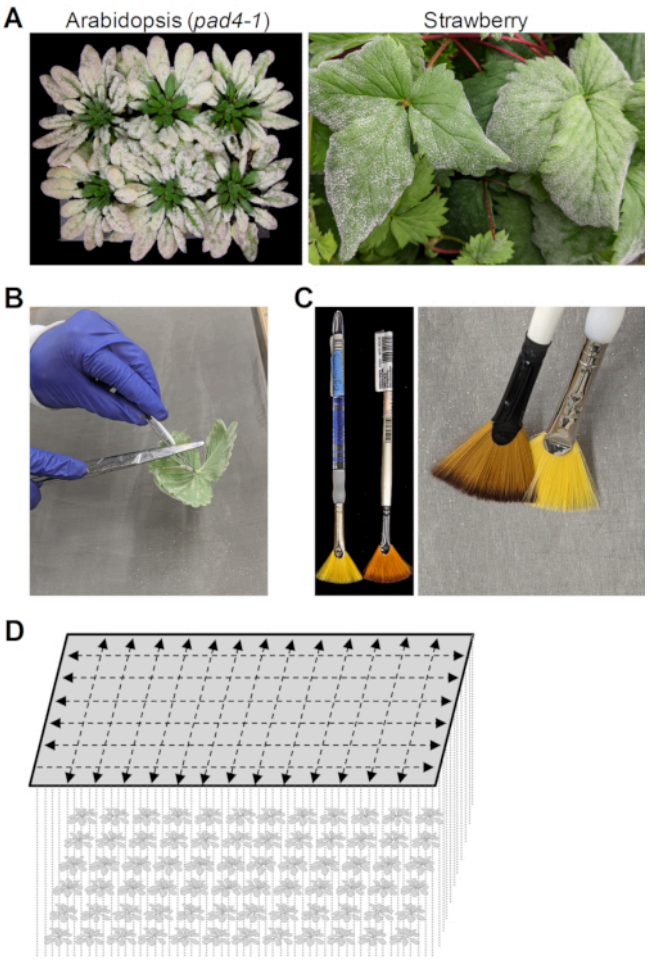

Figure 3. Illustration of the inoculation process. (A) Pictures showing fresh powdery mildew on leaves of indicated plants. **(B)** A picture showing how to shake off spores on the mesh by gently hitting the forceps grabbing the infected leaves. **(C)** Pictures showing two fine fan-blender brushes and gentle brushing of spores on the mesh. **(D)** A schematic drawing showing the directions of spore brushing on the mesh that can help result in even spore distribution on plants in the bottom of the inoculation box. It is important to point out that using fresh spores for inoculation is critical to successful infection. Based on our experience, conidia produced on infected leaves between 8 to 12 dpi are fresh and can be easily dislodged by shaking. Please click here to view a larger version of this figure.

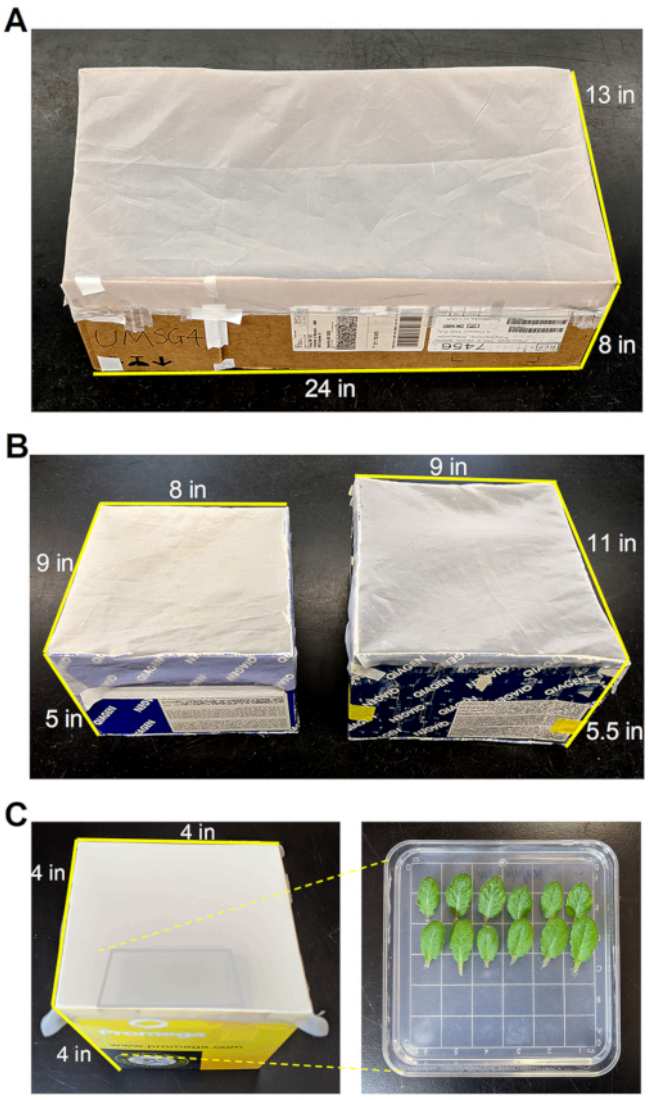

Figure 4. Simple, provisional cardboard inoculation boxes for infection tests. (A) A cardboard box for inoculating plants in a standard flat. **(B)** Medium-sized inoculation boxes for inoculating fewer plants. (**C)** A small inoculation box for inoculating detached leaves in square petri dish plate containing MS-agar medium. Yellow lines highlight the size measurement of the box. Carboard boxes of other sizes can be used if they fit the flats containing plants to be tested. Plastic boxes should not be used because spores can be attracted to plastic surface due to its static electricity. Please click here to view a larger version of this figure.

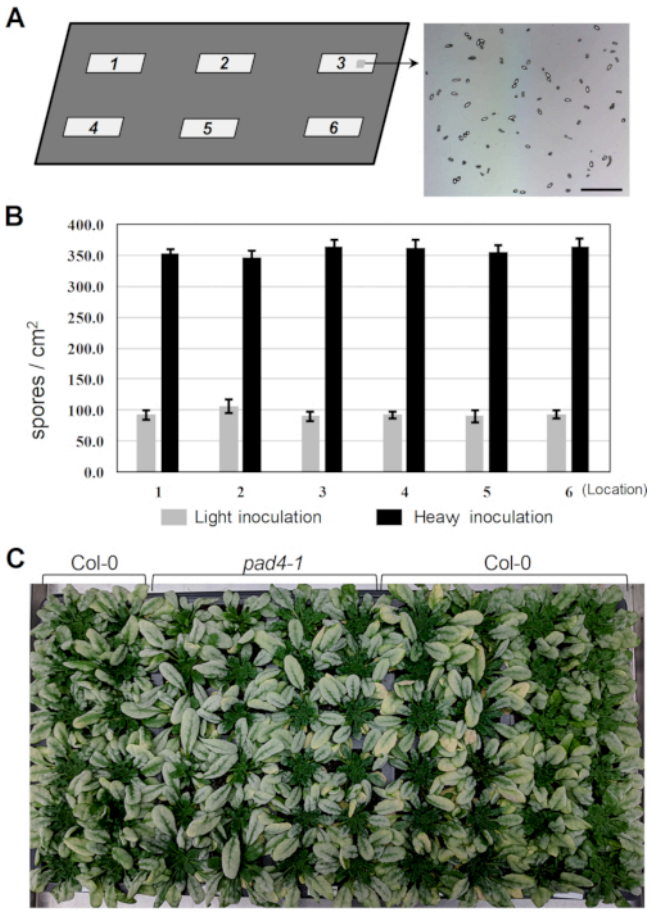

Figure 5. Assessment of inoculation evenness. (A) A schematic drawing showing the positions of six microscopic slides on the bottom of a flat. A representative micrograph on the right shows even spore distribution on the slide after a heavy inoculation. Scale bar = 200 µm. **(B)** A bar chart showing the density of spores distributed in six locations (1 to 6) as shown in (A) after three mock inoculations with spores from four (for light inoculation) or 15 (for heavy inoculation) fully expanded infected leaves of *pad4-1* plants. Spores in an area of 4 x 0.25 cm^2 delimited by a gridded glass cover slip (from ibidi USA Inc. or self-made) on top of each slide were counted. No significant differences were detected in spore density between six slides in each of the two inoculation schemes (Student *t*-test; $p > 0.5$). Error bars indicate SD. **(C)** Representative plants of the *Arabidopsis* Col-0 wild-type and *pad4-1* mutant infected with *G. cichoracearum* UCSC1 at 12 days after a heavy inoculation. Note all *pad4-1* plants showed enhanced disease susceptibility compared to Col-0 plants. Multiple factors determine the inoculation evenness. In general, it is relatively easier to achieve even inoculation when enough inocula are used to reach a density of > 50 spores/cm^2. Fresh conidia dislodged by shaking can be easily disaggregated and individually brushed through the mesh. Old or dead conidia tend to form aggregates, thus are difficult to dislodge and disperse. Please click here to view a larger version of this figure.

Discussion

Our meshed-box-based inoculation method has several advantages over other inoculation methods. First, it can achieve even distribution of spores if operated properly, as demonstrated in **Figure 5**. Second, the use of ~50 µm mesh, plus spore-dislodging by gentle shaking of infected leaves can reduce plant infection by thrips or other plant-infecting insects that are present in source plants. Third, the use of different-sized inoculation boxes for inoculating plants or detached leaves inside the plastic chamber (both of which can be cleaned easily by spraying 75% ethanol) can make more effective use of inoculum and reduce cross contamination. We found that no or very few fungal spores escaped from the inoculation chambers during the entire inoculation process.

A good-quality inoculation box is key for ensuring even inoculation. We found that the standard inoculation box can be used for inoculating young and mature plants of Arabidopsis (**Figure 1**), seedlings of strawberry, sow thistle and *N. benthamiana* (not shown). The height of the box can further increase if plants are taller than five inches to ensure sufficient distance (> 5 in) from the mesh to the plants beneath to allow even spore distribution. The height of the plastic chamber may also increase accordingly to allow adequate space for maneuvering spore dislodging and brushing on the top of the inoculation box.

Even and tight mounting of a ~50 µm mesh of choice to the lid of the inoculation box is important and requires extra care. The pore size is slightly bigger than that powdery mildew spores which are mostly 30-40 µm in diameter. The lid can be cleaned by washing with tap water or spray with 75% ethanol after use. We recommend the use of a 48 µm stainless steel mesh, because the mesh is more durable and will last longer.

The inoculation chamber creates a wind-still environment and minimizes spore escape during spore-dislodging and/or brushing. The chamber is made of transparent plastic glass so that the user can see by the naked eye if the spores dislodged are more or less evenly distributed on the mesh before brushing. This is especially important if a light and even inoculation is required. Brushing gently but quickly in different directions is also important as it can disperse aggregated spores and push them through the pores individually, achieving even distribution after the spores fall and settle down to the bottom of the inoculation box. For easy handling, the inoculation chamber should be placed on a table with a suitable height such that spore-dislodging and brushing can be easily done with hands through the window of the chamber.

The meshed-box-based inoculation can be scaled up or down by using different-sized inoculation boxes. Simple, provisional meshed-inoculation boxes are easy to make and could achieve satisfactory results if used properly. Admittedly, compared to inoculation with the vacuum-operated settling tower method[7], this method may take longer time in spore-dislodging and brushing. Also, for inoculating big and tall plants, the standard inoculation box described here may be too small thus a bigger inoculation box and a larger chamber have to be used to achieve even inoculation. For some plant species such as tobacco and cucumber, detached leaves or cotyledons can be inoculated with this method to assess the disease susceptibility of the whole plant.

Disclosures

The authors have nothing to disclose.

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