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Infection Process Observation of *Magnaporthe oryzae* on Barley Leaves

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[Abstract] Rice blast and wheat blast caused by *Magnaporthe oryzae* is a serious threat to rice and wheat production. Appropriate methods for observing *M. oryzae* infection process are important for study of the fungal infection mechanisms, plant resistance reactions, and host-*M. oryzae* interactions. The rice leaf sheath is commonly used to inoculate *M. oryzae* for observing the infection process, however, this method is a time-consuming and high technical work. Here, we describe an easier solution to observe *M. oryzae* infection process on barley leaves.

Keywords: *Magnaporthe oryzae*, Rice blast fungus, Barley leaf epidermis, Inoculation, Infection process, Plant-pathogen interactions

[Background] The filamentous fungus *Magnaporthe oryzae* can cause destructive rice blast and wheat blast diseases, which can also infect barley (Kohli *et al.*, 2011; Dean *et al.*, 2012). *M. oryzae* has been studied as a model to understand fungal-plant interactions (Yan and Talbot *et al.*, 2016). This fungus initiates its infection by attaching the conidium to host surface, then the conidium germinates and forms a dome-shaped appressorium, by which it can penetrate into host cell for colonization (Wilson and Talbot, 2009). In host cells, the fungus colonizes as a biotrophic manner by forming bulbous and branched infection hyphae to interact with host defense system (Kankanala *et al.*, 2007). *M. oryzae* sequentially invade living host cells and finally transformed into necrotrophic growth, during which the lesions appear and sporulation occurs. In order to study the fungal infection mechanism, or protein functions during infection, or plant defense reaction, it is required to observe cellular infection process of different strains in host cells. A rice leaf sheath method has been commonly used to observe the infection process (Koga *et al.*, 2004), however, this method needs to waste a long time to prepare the rice leaf sheath, and the inoculation and sample preparation need a great deal of experience. Because barley is also the host of *M. oryzae*, and its leaf epidermis is easy for tearing, so we found that barley leaf epidermis method is an effective and simple method to observe the infection process of *M. oryzae*.

Materials and Reagents

1. Petri dishes (6 cm and 9 cm diameters, ASONE)
2. Lens paper (Fisher Scientific)
3. Filter paper (Whatman)
4. Medical gauze (Angyang Medical)

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5. Absorbent paper (Kimberly-Clark)
6. 1.5 ml tubes (Eppendorf)
7. Tips (10 µl, 200 µl and 1,000 µl, Axygen)
8. Blades (Dexter Russell Cutlery, catalog number: 73-C)
9. Glass slides (Fisher Scientific, catalog number: 12-550-343)
10. Coverslips (Fisher Scientific, catalog number: 12-547)
11. Pots (10 cm in diameter and 15 cm in height)
12. Cultivated land soil
13. *Magnaporthe oryzae* strains
    Note: The M. oryzae strains are maintained on dried filter paper pieces stock in -80 °C for long-term storage.
14. Barley seeds (*Hordeum vulgare*, cv E9)
15. Sterilized water (Milli-Q)
16. Boiled oatmeal filtrate
17. Tomato juice
18. Agar (Sigma-Aldrich, catalog number: 17209)
19. Tween 20 (Sigma-Aldrich, catalog number: P9416)
20. Oatmeal tomato agar (OTA) solid media (see Recipes)
21. 0.025% Tween 20 (see Recipes)

Equipment

1. Scissor, tweezers, spreader
2. Pipettes (Eppendorf, catalog numbers: 3124000016 [0.5-10 µl], 3124000032 [20-200 µl], and 3124000075 [100-1,000 µl])
3. Incubation chamber (28 °C) for fungal growth and barley germination (Biolab Scientific, model: BIFG-101)
4. Hemocytometer (Marienfeld, catalog number: 0650030)
5. Greenhouse capable of temperature and humidity control for growing barley
6. Optical microscope (Olympus, model: CX23)
7. Fluorescence microscope (Leica Microsystems, model: Leica DM2500)
8. Juice extractor
9. Autoclave

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Procedure

A. Preparation of *M. oryzae* (Figure 1)

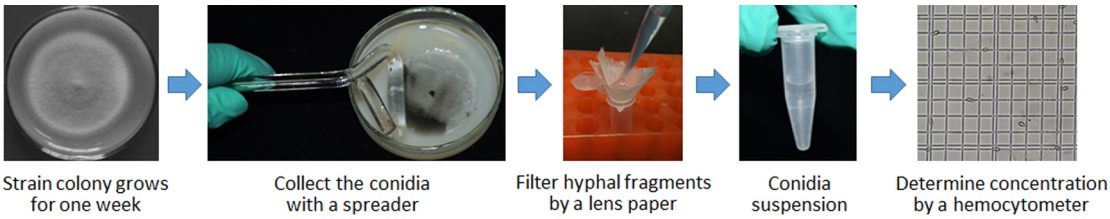

Figure 1. Preparation procedure for conidia suspension of *M. oryzae*

1. Inoculate the *M. oryzae* strains into solid OTA (Recipe 1) plates (6 cm diameter) and incubate for one week in an illumination incubator at 28 °C.
2. Add 2 ml sterile distilled water containing 0.025% Tween 20 (Recipe 2) to each OTA plate and scrape with a spreader to harvest conidia. Use two layers of sterile lens paper to filter and remove hyphal fragments. Upon filtering, brief vortex by pipetting to re-suspend conidia.
   Note: Tween 20 will help to increase water hydrophobic property, therefore promote the formation of the water drops without collapse.
3. Determine the conidia concentration by using a hemocytometer under an optical microscope, and adjust the final concentration to 1 x 10^5 per ml of 0.025% Tween 20 sterile water.

B. Preparation of barley leaves
1. Put barley seeds (around 50 seeds) into a Petri dish with an appropriate volume of sterile water and place the Petri dish in an incubation chamber at 28 °C for 12 h.
2. Remove the water, and wash the seeds for several times with sterile water, then hung up in one layer gauze to dry for 6 h.
3. Put the seed into the Petri dish (9 cm diameter), and immerse the seeds with an appropriate volume of sterile water, then cover several layers of wet gauze. Place the Petri dish in an incubation chamber for 12-18 h at 28 °C.
4. After the seeds germinate, transfer them into pots (10 cm in diameter and 15 cm in height) containing 80% volume of cultivated land soil (20-30 seeds per pot). Place the pots in a greenhouse for growth at room temperature. Water the pot every two days to keep the soil wet. After one week, the barley leaves can be used for inoculation (Figure 2).

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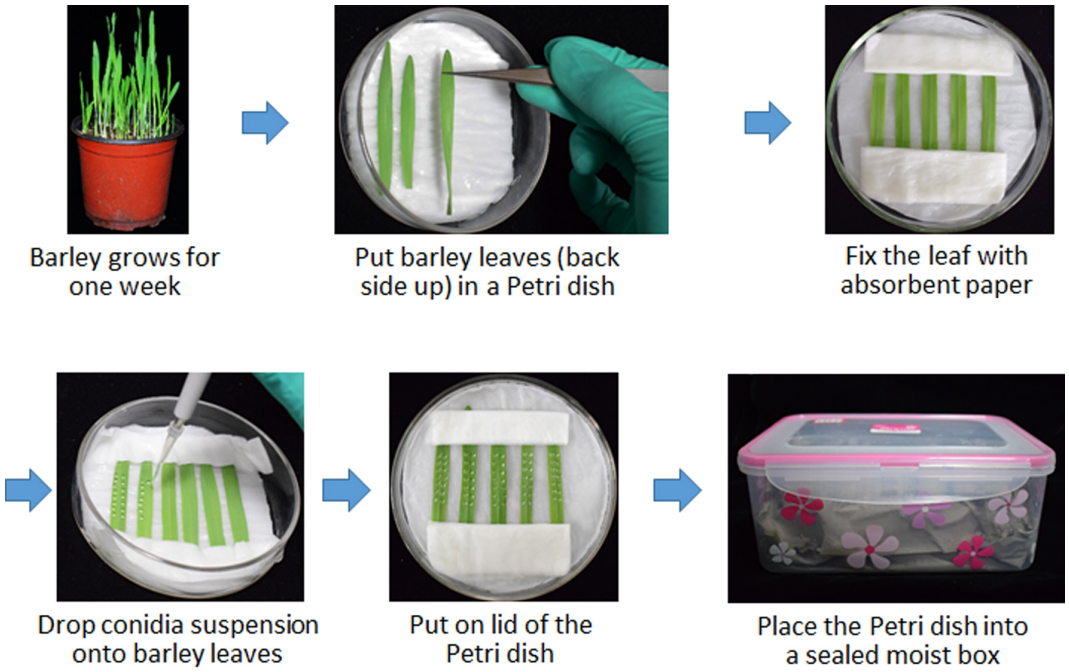
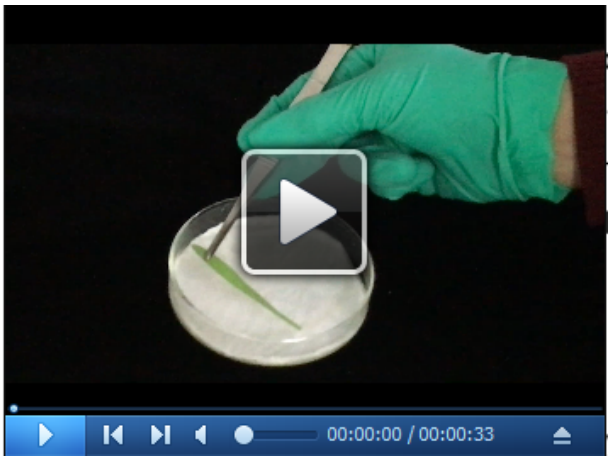


Figure 2. Procedure for inoculation of *M. oryzae* on barley leaves

C. Inoculation of barley with *M. oryzae* (Figure 2)

1. Cut the barley leaves from the base with a scissor, then put them (back side up) in a Petri dish (9 cm diameter), which have been covered with two layers of water immersed absorbent paper. Fix the leaf base and tip with water immersed absorbent paper, and keep leaf flat (Video 1).
   Note: When fix the leaves base and tip with water immersed absorbent paper, keep the leaves dry and don't touch the leaves with fingers or any other materials.



Video 1. Put barley leaves into the Petri dish and fix with water immersed absorbent paper

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2. Drop the conidia suspension onto barley leaf beside the vein using a pipette (0.5-10 μl range) (Video 2).
   Note: Keep each drop with a diameter of around 2-3 μm and without collapse, if the drop is too large or collapsed, it may tend to the formation of aerial mycelium, but not appressorium. Keep the tips away from the epidermis could avoid collapse. Re-suspending conidia by shaking up and down of the tubes every time before absorbing the conidia suspension.

Video 2. Drop conidia suspension onto the barley leaves

3. Put on the lid, cover the Petri dishes with wetted absorbent papers, and place them into a sealed moist box. Then put the box in a dark incubator at 28 °C.

D. Infection process observation (Figure 3)
   1. Takeout the barley leaves with a tweezer at different time points after inoculation. Use a blade to cross cut the leaf from up to down, at the tip side or base side. Keep the lower epidermis still connected, and subsequently tear down the lower epidermis (Figure 3A, Video 3).
      Note: During this process, try best not to touch the water drops, which could break the infection structures. Recommend observing penetration pegs and primary infection hyphae at 18 hpi, and secondary infection hyphae at 24-30 hpi.

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Video 3. Tear down the barley lower epidermis and put onto a glass slide

2. Put the lower epidermis onto a glass slide, add some water to immerse the epidermis, then cover it with a coverslip.
3. Observe the infection processes of *M. oryzae* in the cells of epidermis under a microscope, take images and calculate formation ratios of different infection structures (Figure 3A). For each time, more than 50 conidia should be calculated.
4. Observe the subcellular localization of GFP-tagged proteins in infection structures under a fluorescence microscope (Figure 3B).
   Note: During observing, remember to add water when the sample is dried.

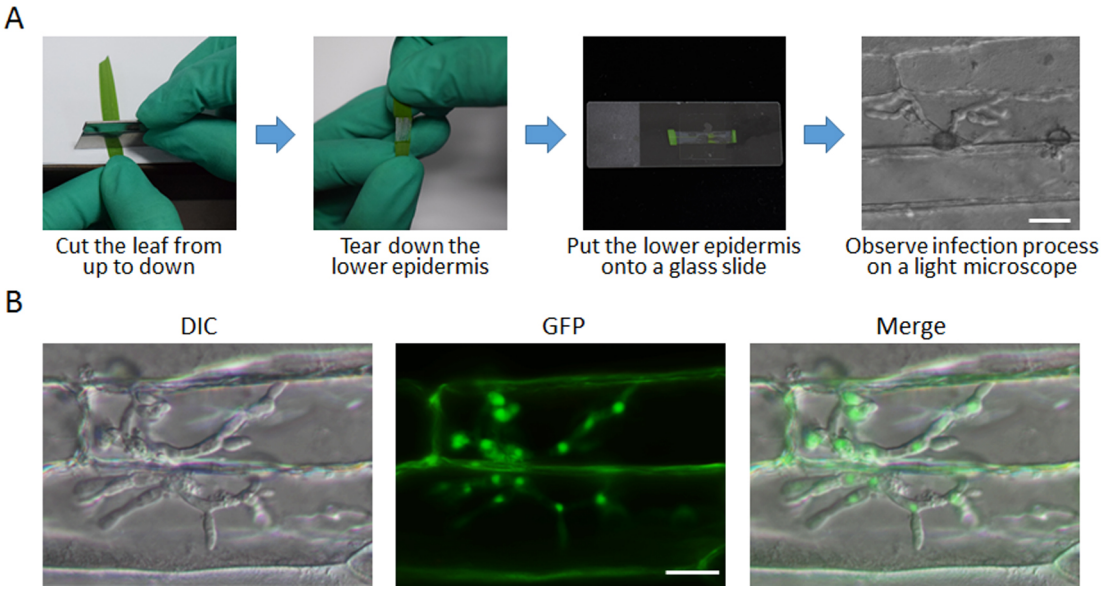

Figure 3. Procedure for observing of different samples. A. Procedure of observing the infection process. B. Observation of protein subcellular localization. DIC, differential interference contrast; GFP, green fluorescent protein. Scale bars = 20 μm.

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Data analysis

All information about data processing, statistical tests, replicates and independent experiments were already included in the original research paper (Chen *et al.*, [2014], N-glycosylation of effector proteins by an α-1,3-mannosyltransferase is required for the rice blast fungus to evade host innate immunity. *Plant Cell* 26(3):1360-1376. doi: 10.1105/tpc.114.123588).

Notes

1. Plant healthy is very important for the success of the infection assay. Barley plant should grow under adequate sunshine, and avoid soil drought. Otherwise, the barley epidermis can't be well infected with *M. oryzae*.
2. Accurately addition of 0.025% Tween 20 into the sterile water is important for successful inoculation. The tips should be cut off before absorbing the reagent. Absorb the Tween 20 slowly and the adhering reagent on tips should be removed.
3. To prevent the drops from collapse during inoculation steps, the Petri dish should be moved as slowly as possible.

Recipes

1. Oatmeal Tomato Agar (OTA) solid media (1 L)
   40 g boiled oatmeal filtrate
   150 ml tomato juice
   20 g agar
   a. Boil the oatmeal in 800 ml distilled water for 30 min, then filtrate the mixture by double-layer gauze
   b. Extract tomato juice by an extractor, then also filtrate the mixture by double gauze. Mix 150 ml tomato juices with boiled oatmeal filtrate, then add water to 1 L
   c. Add 20 g agar, and autoclave for 40 min
2. 0.025% Tween 20
   Add 250 µl Tween 20 in 800 ml sterilized distilled water and adjust the volume to 1 L

Acknowledgments

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