Video Article

# The Plant Infection Test: Spray and Wound-Mediated Inoculation with the Plant Pathogen *Magnaporthe Grisea*

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## Abstract

Plants possess a powerful system to defend themselves against potential threats by pathogenic fungi. For agriculturally important plants, however, current measures to combat such pathogens have proved too conservative and, thus, not sufficiently effective, and they can potentially pose environmental risks. Therefore, it is extremely necessary to identify host-resistance factors to assist in controlling plant diseases naturally through the identification of resistant germplasm, the isolation and characterization of resistance genes, and the molecular breeding of resistant cultivars. In this regard, there is need to establish an accurate, rapid, and large-scale inoculation method to breed and develop plant resistance genes. The rice blast fungal pathogen *Magnaporthe grisea* causes severe disease symptoms and yield losses. Recently, *M. grisea* has emerged as a model organism for studying the mechanisms of plant-fungal pathogen interactions. Hence, we report the development of a plant virulence test method that is specific for *M. grisea*. This method provides for both spray inoculation with a conidial suspension and wounding inoculation with mycelium cubes or droplets of conidial suspension. The key step of the wounding inoculation method for detached rice leaves is to make wounds on plant leaves, which avoids any interference caused by host penetration resistance. This spray/wounding protocol contributes to the rapid, accurate, and large-scale screening of the pathotypes of *M. grisea* isolates. This integrated and systematic plant infection method will serve as an excellent starting point for gaining a broad perspective of issues in plant pathology.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57675/

## Introduction

Rice blast, caused by *M. grisea*, is one of the most serious diseases for rice varieties worldwide[1,2]. The process by which *M. grisea* infects host plants includes a conidia production and surface attachment, a conidia germination and appressorium formation, a formation of the penetration peg and infectious hypha differentiation, and a disease spread[3]. All these stages are common in many other plant pathogenic fungi, and, indeed, a blockade of any single stage prevents the infection of host plants. Owing to its economic importance and genetic tractability, *M. grisea* has emerged as a model organism for studying the mechanisms of plant-fungal pathogen interactions[1,4]. Therefore, studying the molecular basis of these developmental stages in *M. grisea* will help to elucidate the molecular mechanisms underlying fungal pathogenicity and the identification of candidate target genes for screening and designing novel fungicides[5].

Recent reports concerning *M. grisea* infection have focused on the molecular mechanisms of the pre-penetration stages, especially the conidiation, the appressorium formation, the penetration pegs, and the infectious growth[3,6]. Therefore, it is essential to develop a detailed protocol to test *M. grisea* infection. Herein, we present a detailed method for an infection test that utilizes spray-mediated infection assays with a conidial suspension and the inoculation of wounds with mycelial plugs of *M. grisea*. In this report, the protocol focuses on the culture of strains, the preparation of the conidiation solution for spraying, and the mycelial plug-mediated inoculation of plants with *M. grisea*. These steps are described in detail below, and a schematic view showing the entire workflow of the method and a typical lesion are shown in **Figures 1** and **2**, respectively.

## Protocol

### 1. Spray Inoculation with a Suspension of *M. grisea* Conidia

1. **Fungal culture for *M.grisea***

1. Prepare the oatmeal tomato agar (OTA) culture medium for fungal strains.
2. Weigh 30 - 50 g of oatmeal, add this to 800 mL of distilled/deionized water ($ddH_2O$) and boil the mixture for 30 min in the electric pot.
3. Filter the boiled oatmeal juice into the beaker through a piece of gauze.
4. Add 150 mL of tomato juice and 20 g of agar to the filtrate in the beaker and add $ddH_2O$ up to 1,000 mL.

2. **Preparation of experimental materials**
   1. Soak about 50 seeds of rice (*Oryza sativa*) cultivar Lijiangxintuan-heigu (LTH) in $ddH_2O$ for 3 d or soak about 50 seeds of barley (*Hordeum vulgare* cv Golden Promise) in $ddH_2O$ for about 1 d.
   2. Wrap the seeds of rice or barley in moist gauze and germinate them on moist filter paper in about 30 Petri dishes with a diameter of 10 cm x 10 cm at 28 °C. The rice seed germination time is about 2 - 3 d, the barley seed germination time is about 2 d. The relative humidity in the greenhouse is approximately 70%.
   3. Plant the seedlings of the rice or barley in pots using autoclaved potting soil and watering and then cover them with a layer of vermiculite.
   4. Place the plants in a suitable glasshouse or growth cabinet at 25 °C for about 1 ~ 2 weeks.
   5. Cut 3 layers of filter paper in circles with sterile surgical scissors (each circle should have an 8 cm diameter) and place them onto 100 mm sterile plastic plates.
   6. Add $ddH_2O$ to each dish to soak the filter paper.
   7. Make sure the filter paper is completely wet but add no extra water.
   8. Remove any excess water with a vacuum pump.
   9. Place 2 sterile toothpicks into the culture dish to support the rice/barley leaves; space the toothpicks ~2 - 3 cm apart.
   10. Collect the leaves of the rice 2 weeks after sowing the seeds or take the leaves of the barley 7 d after sowing the seeds.
   11. Using 4- to 6-leaf seedlings of rice/barley, cut the lower part of the stem at ~5 cm from the top and collect the leaves.

3. **Spray inoculation protocol**
   1. Culture the fungal strain on OTA plates in a thermostatic incubator (25 °C) for ~4 d.
   2. Add ~2 mL of $ddH_2O$ using a 0.5 - 5 mL pipette to each 4-day-old plate.
   3. With an inoculation loop, scrape the mycelia of the *M. grisea* wild-type strain and the mutant strain into mycelia debris.
   4. Collect the mycelia debris and transfer it to a new OTA plate. Take the mycelia debris and blow dry in the clean bench.
   5. Cover the plate with 3 layers of gauze to ensure the humidity required for the growth of the conidia in a greenhouse at 25 °C in the day (14 h) and 23 °C in the night (10 h) for 24 - 48 h.
   6. Add 2 mL of $ddH_2O$ to each dish and scrape the conidia gently with sterile cotton swabs followed by a filtration through 2 layers of lens paper. Be careful not to scratch the surface of the culture medium.
   7. Transfer the conidia suspension into a new 50 mL tube with a 100 - 1,000 µL pipette.
   8. Centrifuge for 5 min at a minimum of 5,000 x g at 25 °C.
   9. Remove the supernatant and resuspend the pellet to give 2 x $10^4$ conidia per mL in a 0.025% (v/v) Tween-20 solution. The Tween-20 solution is usually about 10 - 20 mL.
   10. Pour the spore suspension into a hand-held sprayer.
   11. Spay about 10 mL of the conidial suspension onto the rice leaves of 2-week-old rice seedlings or barley leaves of 7-day-old barley seedlings and incubate them at 25 °C in a dark, humid chamber for ~24 h. Spray the control plants with the 0.025% (v/v) Tween-20 solution.
   12. Transfer the leaves into another moist chamber under fluorescent light at 25 °C for a photoperiod of 12 h.
   13. Record the disease symptoms at 5 d after the inoculation. Examine the diseased rice/barley blades of ~6 cm in length. The evaluation standard is according to the scoring system for blast resistance of the International Rice Research Institute (IRRI). The details of the scoring system for blast resistance are shown in **Table 1**.
   14. Photograph the leaves to evaluate the infection of the tested strains. The infection was assessed by the number of lesions per 3.6 $cm^2$.

## 2. Wounding Inoculation with Mycelium Cubes or Droplets of Conidial Suspension of *M. grisea*

1. Using an anatomical needle, scrape three 2 - 3 cm long wounds in the main veins of detached rice/barley leaves; take care not to penetrate the leaves.
2. Put the scraped leaves on toothpicks and spray a 0.02% (v/v) Tween-20 solution on the leaves to form a layer of droplets.
3. Cut a 0.5 cm x 0.5 cm mycelial plug for each *M. grisea* strain (wild-type, mutant, complement strains, or other test strains) from an OTA plate.
4. Put the mycelial plugs or 25 µL droplets of conidial suspension onto the wounded leaves and incubate the leaves at 25 °C in a humid chamber for 3 - 8 d.
5. Examine the lesions at 5 - 7 d post-inoculation. The method of examination is the same as it was for the spray inoculation method (see step 1.3.13).
6. Examine the diseased rice/barley blades of ~6 cm in length and photograph them to evaluate the infection of the tested strains. The infection was assessed by the number of lesions per 3.6 $cm^2$.

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## Representative Results

The entire workflow for the technique is shown in **Figure 1**. The plant infection assays were performed on 14-day-old susceptible rice seedlings (*O. sativa* cv CO-39) or susceptible 7-day-old barley leaves (*H. vulgare* cv Golden Promise)[7,8,9]. To test for an infection on the rice leaves, a conidial suspension (1.0 x $10^5$ spores/mL) of the *M. grisea* wild-type strain P131 and the Com1 deletion mutant strain were prepared and then sprayed onto the leaf sheaths of the 14-day-old susceptible CO-39 seedlings, which were then kept in a moist chamber for 5 d[10]. The P131-inoculated CO-39 leaves displayed the typical robust lesions of rice blast, but the Com1-inoculated leaves showed obvious infection defects and could not elicit a full infection (**Figure 2A**). The wild-type strain P131 is a strain obtained by You-Liang Peng in 1988[18]. The MoKMT2H null mutant (*ΔMoKMT2H*) was obtained by Cao *et al.* in our lab using a target gene replacement strategy[11]. The *Com1* mutant was isolated by Yang *et al.* in You-Liang Peng's lab[10].

To check whether the *M. grisea ΔMoKMT2H* could infect the host cells through wounds, abraded leaves of wild-type rice plants were inoculated with mycelial plugs of *ΔMoKMT2H via* the spray method or wounding method[11]. The leaves that were spray-inoculated with *ΔMoKMT2H* revealed no obvious defects in the *ΔMoKMT2H* infection compared with the P131/wild-type strain; however, obvious defects were observed for leaves inoculated with *ΔMoKMT2H via* wounds (**Figure 2A**). To further test the plant infection with barley leaves, healthy or wounded leaves (cv Golden Promise) were inoculated with conidial droplets or mycelial plugs, respectively, of Com1, *ΔMoKMT2H*, or P131. At 5 d post-inoculation, typical rice blast lesions had fully developed on the leaves inoculated with either the *ΔMoKMT2H* or P131 strain, whereas fewer and smaller lesions were found on the leaves inoculated with the Com1 mutant (**Figure 2B**).

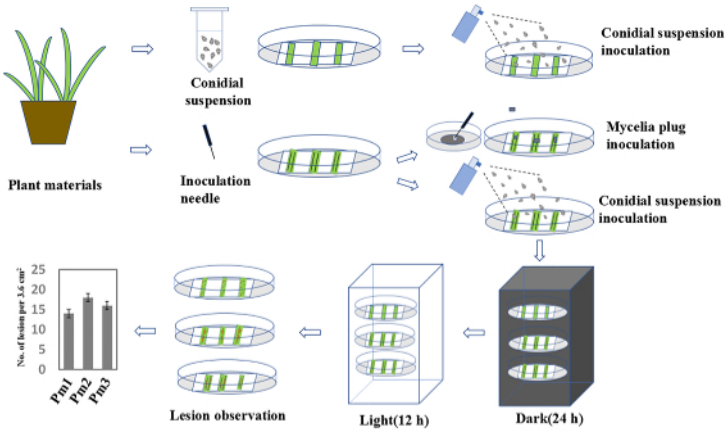


**Figure 1: A scheme illustrating plant infection.** Plant seeds were germinated in soil in plastic pots (50 $mm^2$ x 50 mm deep, with a drainage hole), two seeds per pot, and the seedlings were grown in a greenhouse. The culture and inoculations of the blast fungus *M. grisea* were grown on OTA plates. For the conidia spraying inoculation method, the conidia suspension was suspended in a 0.02% (v/v) Tween-20 solution and sprayed onto the rice/barley plants. For the scratched inoculation method, the scraped rice/barley plant leaves were put on plastic plates and then inoculated by a mycelial plug or the conidia suspension. And then, all the treated plant leaves were dark-cultured for 24 h and light-cultured for 12 h. Finally, the number of colonies within units of 3.6 $cm^2$ was recorded. Please click here to view a larger version of this figure.

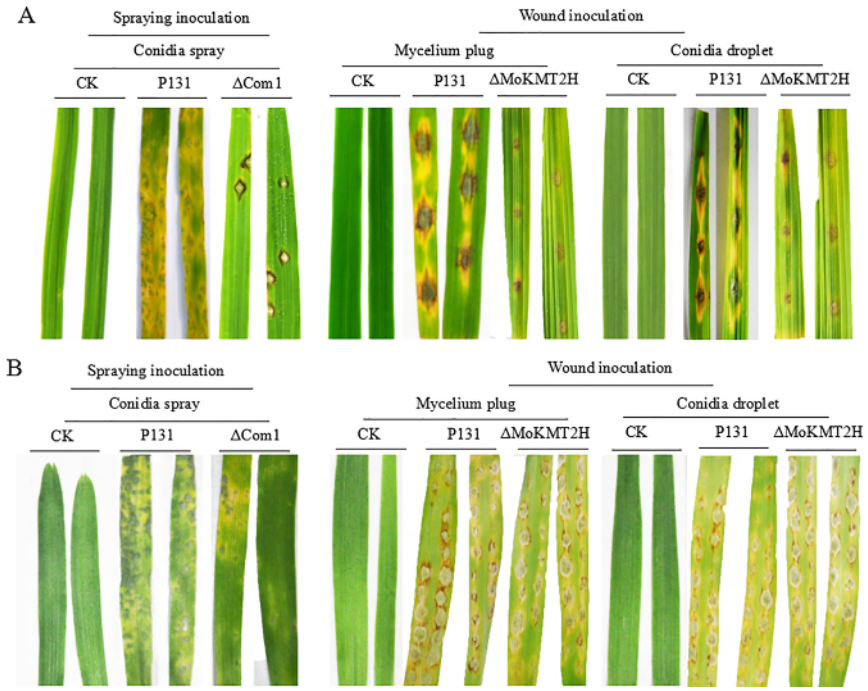


**Figure 2**: ***Com1* and *MoKMT2H* are required for a conidium formation and pathogenicity on rice leaves**. (**A**) This panel shows the inoculation of rice leaves *via* conidia spray (left) or mycelial plug (right) with conidia from the *M. grisea* wild-type P131, mutant *Com1*, or *ΔMoKMT2H*. Typical leaves were observed 7 d after the mycelial plug-mediated inoculation, which was conducted on abraded rice leaves. Typical lesions were observed 5 d after the mycelial plug-mediated inoculation. (**B**) Barley leaves were inoculated *via* conidia spray (left) or mycelial plug (right). As a mock treatment control, the same volume of a 0.025% (v/v) Tween-20 solution was sprayed. For the wound inoculation, the figure of P131 has been modified from Cao *et al.*[11]. The *MoKMT2H* in the ascomycete fungi is a functional homolog of Ash1, which is implicated in H3K4 and H3K36 methylation[11]. The bar = 1 cm. The *Δcom1* mutants were significantly reduced in virulence on the rice and barley seedlings[10]. Please click here to view a larger version of this figure.

| Scale | Description |
|---|---|
| 1 | Small brown specks of pin-point size |
| 2 | Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter ,with a distinct brown margin .Lesions are mostly found on the upper leaves |
| 3 | Lesion type is the same as in 2, but significant number of lesion are on theupper leaves |
| 4 | Typical susceptible blast lesions, 3 mm or longer, infecting less than 4% of theleaf area |
| 5 | Typical susceptible blast lesions, 3 mm or longer, infecting less than 4-10% ofthe leaf area |
| 6 | Typical susceptible blast lesions, 3 mm or longer, infecting less than 11-25% ofthe leaf area |
| 7 | Typical susceptible blast lesions, 3 mm or longer, infecting less than 26-50% ofthe leaf area |
| 8 | Typical susceptible blast lesions, 3 mm or longer, infecting less than 51-75% ofthe leaf area, many leaves dead |
| 9 | Typical susceptible blast lesions, 3 mm or longer, infecting more than 75% ofthe leaf area |

**Table 1: A scoring system for blast resistance.** The table is cited from the International Rice Research Institute (IRRI).

## Discussion

Plant disease resistance genes play an essential role in preventing infections by pathogens, including fungal pathogens[1,12]. Rice blast has been used as a model to understand the nature of pathogen population structures and to identify plant resistance genes[4]. Therefore, it is necessary

to examine the disease resistance genotype and avirulence genotypes of the main varieties of agricultural plants on a large scale to identify disease-resistant plants that can be cultivated continuously. Furthermore, the advantages of avirulence genotypes of field pathogens necessitate the rational distribution of the different resistant genotypes of host varieties[12,13]. However, current inoculation methods commonly interfere with the screening for resistance and pathogen identification[14,15,16,17].

Here, we present a rapid and accurate method to test for plant infection. This inoculation method is suitable for the identification of resistant plants during breeding trials and of the phenotype of a progeny population during the cloning of resistance genes. Here, we established a method for inoculating wounded plant leaves with *M. grisea*. Because the host resistance plays a major role in preventing the spreading of a pathogen, this *in vitro* method, which produced wounds on the tested rice leaves and inoculated the rice blast on the wound, is convenient for creating an infection directly into the leaves without having to penetrate the leaf epidermis and the epidermal cell wall. Furthermore, this inoculation method is suitable for different leaf ages. The inoculation results are stable and accurate. The method of wounding inoculation can be used for inoculation with mycelia to identify the pathogenicity of fungal strains which produce only low amounts of conidia.

This protocol will further contribute to our understanding of the pathogenic mechanisms that are conserved in fungi, as well as the pathogen-specific factors that permit a fungus to resist and suppress the innate immunity of its host[13]. However, the wound inoculation is not applicable when trying to determine the rate of a conidial invasion and mycelia expansion. But in the natural state, the spray inoculation method can better reflect the pathogenicity of a pathogen. The spray inoculation method is easy to operate and helps to improve work efficiency. Taken together, these results indicating an accurate and stable inoculation method are necessary for screening plant resistance genes and determining pathogenicity.

## Disclosures

The authors have nothing to disclose.

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