

# Rice blast infection of *Brachypodium distachyon* as a model system to study dynamic host/pathogen interactions

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**Interactions between plants and compatible fungal pathogens are spatially and temporally dynamic, posing a major challenge for sampling and data analysis. A protocol is described for the infection of the model grass species *Brachypodium distachyon* with *Magnaporthe grisea* (rice blast), together with modifications to extend the use to rice and barley. We outline a method for the preparation of long-term stocks of virulent fungal pathogens and for the generation of fungal inoculants for challenge of host plants. Host plant growth, pathogen inoculation and plant sampling protocols are presented together with methods for assessing the efficiency of both infection and sampling procedures. Included in the anticipated results is a description of the use of metabolite fingerprinting and multivariate data analysis to assess disease synchrony and validate system reproducibility between experiments. The design concepts will have value in any studies using biological systems that contain dynamic variance associated with large compositional changes in sample matrix over time.**

## INTRODUCTION

Over the past few decades, academic and industrial researchers have developed a range of technologies to help understand the basic principles of fungal pathogenicity and plant susceptibility. By investigating the adaptations required to exhibit a pathogenic lifestyle it becomes possible to identify new potential strategies for plant protection. The importance of cereal diseases is highlighted by the fact that several serious pathogens affecting gramineae species were among the first wave of filamentous fungal genomes to be sequenced systematically<sup>1</sup>. With the parallel development of genomics resources, model system status has been proposed recently for several fungal pathogens including *Magnaporthe grisea* (rice blast), *Fusarium graminearum* (wheat head blight) and *Ustilago maydis* (maize smut)<sup>2–4</sup>. *F. graminearum* and *U. maydis* are specialized pathogens adapted to infect, respectively, the emerging floral tissues and the developing seed heads of host plants. In contrast, *M. grisea* develops invasion structures and has a lifestyle typical of a much wider range of cereal pathogens which directly penetrate leaf tissues through the cuticle<sup>2,5</sup>. This fact, in combination with its genetic tractability, wide host range and ability to develop infection structures *in vitro*, have focused international effort on developing *M. grisea* as a model functional genomics system representative of a wide range of fungal pathogens<sup>2,5</sup>.

Study of the genetics and molecular biology of disease resistance and particularly the mechanism(s) of the hypersensitive response has provided major insight into how potential hosts recognize and contain invading microbes<sup>6–8</sup>. In contrast, studies of compatible plant pathogen interactions at the molecular level are not as widely reported<sup>9–13</sup>. The early phases of disease establishment dictate whether or not the pathogen will be successful in its colonization attempts and often several days elapse before lesions are visible to the naked eye. At the macroscopic level, the contribution of pathogen biomass to the overall sample matrix increases during

tissue colonization; where pathogen-challenged leaf material is composed of a heterogeneous mosaic of developing lesions, adjacent infected cells and noninfected areas. These spatially and temporally dynamic zones of interaction pose major sampling challenges in any studies. Therefore, it is crucial to develop validated sampling protocols to ensure that infections are as synchronous as possible and reproducible between experiments. In addition, it is important to ensure that the infection and harvest techniques applied can cope with the large and dynamic changes that occur during the infection process.

In this protocol, we describe an experimental system comprising a compatible interaction between a model fungal pathogen (*M. grisea*) and a model grass species (*Brachypodium distachyon*)<sup>14</sup>, which is being developed as a resource for studies on fungal pathogenicity and susceptible interactions at all 'omics' levels. This protocol is suitable for any strain of *M. grisea* (whether compatible or incompatible on a specific host) and it also includes modifications of core method to extend the use to two major crops (barley and rice). The general principles regarding pathogen maintenance, generation of a virulent inoculum and challenge of host plants may be extended to several other fungal species that can grow and sporulate *in vitro* including *Alternaria*, *Botrytis*, *Colletotrichum*, *Mycosphaerella*, *Fusarium*, *Rhynchosporium* and *Cochliobolus*<sup>15–18</sup>.

## *M. grisea* as a model plant pathogen

Rice blast disease causes the destruction of enough rice each year to feed 60 million people<sup>19</sup> and is difficult to control once established. As a consequence of its economic importance, *M. grisea* is a target organism for fungicide development by agrochemical companies throughout the world. However, the impact of *M. grisea* is not only felt by rice growers but also by other crop growers, because some strains are able to attack other cereals such as wheat, barley and

**TABLE 1** | Time course of the major events during pathogenesis by *Magnaporthe grisea*.

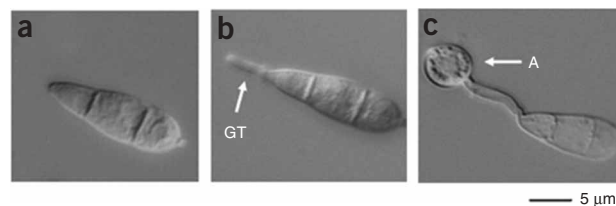
Time (h)	Developmental event
0	Conidia land on rice surface. Spore-tip mucilage released and spore adhesion
0.5–1.5	Conidia germinate. Elongation of the germ tube
2–4	Tip growth ceases. Hooking, apical expansion and formation of the appressorium. Septum forms at base of appressorium. Bottom cell wall of appressorium thins and new cell-wall layers form. Membrane cisternae and vesicles abundant
4–8	Melanization begins. Membrane cisternae and vesicles absent. Appressorium pore becomes well defined
16–24	Granular substance (possibly an adhesive) accumulates at interface with rice leaf Glycogen rosettes abundant. Pore ring is clearly seen under rim of appressorium
31–24	Formation of pore-well overlay. Glycogen rosettes nearly absent. Greatest recorded appressoria turgor pressure (26–46 h). Emergence of penetration peg, ingress through cell wall and entry into epidermal cell
31	Primary infection hyphae forms in epidermal cell
48	Bulbous secondary hyphae form and spread to adjacent epidermal cells
72	Secondary hyphae spread into mesophyll cells. Some browning and autofluorescence of mesophyll cells is often seen. Up to 10% of total biomass is fungal
96	Lesions are now visible to naked eye on rice leaves Longer hyphae visible at lesions margins
144	Conidiation initiates under conditions of high humidity Lesions begin to coalesce. Hyphae fill leaf tissue Conidiation continues under conditions of high humidity

pearl millet, as well as some turf grasses, causing serious disease outbreaks in South America, Africa and the United States<sup>2,5</sup>. *M. grisea* is well adapted to attack and penetrate its host and all aerial parts of the plants are subject to invasion. In addition, it was recently shown that *M. grisea* has the capacity to infect roots and spread systemically in the plant tissue of susceptible hosts<sup>20</sup>. The infection process is very similar in all susceptible hosts and a summary of various major events during disease progression and representative visible aspects of the invasion process and disease establishment are presented in **Table 1**.

*M. grisea* is a filamentous ascomycete and an excellent model organism for the study of phytopathogenic fungi and interactions with hosts<sup>2,5</sup>. The fungus can be readily transformed and as a consequence targeted gene replacement is a routine procedure, allowing gene functional studies to be performed. In addition, spatial analysis of gene expression and protein localization are routinely carried out with reporter genes, and the cell biology of plant infection is well described<sup>2,5</sup>. The predominant form of reproduction by *M. grisea* is asexual, using mitotically produced spores (conidia) that emerge from lesions on aerial plant parts. A single lesion can produce 2,000 to 6,000 conidia per day for up to 24 d, making possible multiple cycles of infection and reproduction during one growing season<sup>21</sup>. The initial stages of infection are relatively nonspecific and permit colonization of host species other than rice, including the model grass species *B. distachyon*<sup>14,22</sup>. *M. grisea* has a relatively small genome of ~40 Mb contained within 7 chromosomes<sup>23</sup>. With the *M. grisea* genome sequence now available, international efforts are underway to define genes that are expressed during spore adhesion, germination and appressorium formation, as well as making it possible to recognize pathways central to infection related development (<http://www.broad.mit.edu/annotation/fungi/magnaporthe/>). *M. grisea* data are well represented in the COGEME (The Consortium for Functional Genomics of Microbial Eukaryotes) relational database that is now very widely used by the fungal research community<sup>24,25</sup>. In addition, *M. grisea* is not an obligate parasite and can be grown easily in a variety of culture formats. The small genome and undemanding growth

habits have allowed the development of efficient technologies for mutagenesis and classical genetic analysis making it one of the most genetically tractable plant pathogenic fungal species.

The conidia of *M. grisea* initiate infection once they land on a leaf surface (**Fig. 1a**). These spores germinate immediately upon contact with the rice leaf, and adhere tightly to the hydrophobic surface by means of spore tip mucilage that is released from the apex of the spore<sup>26</sup>. Germination proceeds by extension of a narrow germ tube (**Fig. 1b**) which emerges from the conidium within an hour of its landing on the leaf surface<sup>19,26</sup>. Within 4 h, the germ tube starts to swell at its apex, and flattens against the surface of the rice leaf. The germ tube apex then develops into a swollen dome-shaped cell, called the appressorium (**Fig. 1c**) that adheres tightly to the leaf surface<sup>19,27</sup>. After the generation of high turgor pressure, a penetration peg breaches the host epidermis allowing ingress of primary infection hyphae. At the same time, the three-celled conidium undergoes autophagic programmed cell death, which is a pre-requisite for appressorium function and subsequent plant infection<sup>28</sup>. Bulbous secondary infection hyphae subsequently develop and spread to adjacent epidermal cells, using plasmodesmata as a conduit for cell-to-cell movement<sup>29</sup>. Up to this stage, the pathogen infection strategy is biotrophic and very similar in developmental program to that exhibited by many important obligate biotrophic fungal pathogens, such as powdery mildew<sup>30,31</sup>.



**Figure 1** | Viability testing of *Magnaporthe grisea* conidia by measuring germination and appressorium production *in vitro*. (a) Conidia hydration at 0.25 h; (b) germ tube (GT) emergence after 6 h; (c) appressorium (A) formation after 24 h. Bar = 5 µm.

Lesion development ensues in the host once hyphal expansion spreads into mesophyll cells (typically 72 h after infection). Rice blast infection in compatible hosts exhibits many characteristics of disease progression that are common to a wide range of fungal pathogen/host interactions (such as appressorium development, intracellular plant colonization and lesion generation) and therefore it is an excellent model system for the study of plant fungal diseases.

### ***B. distachyon* as a model plant species**

*B. distachyon* has been described as a new model system for functional genomics in grasses for several reasons<sup>14</sup>, as well as being a host for *M. grisea*. It has a relatively small genome with very little repetitive DNA, exhibits a low degree of DNA methylation; the *Brachypodium* nuclear genome when compared to *Arabidopsis* was indistinguishable in size, which makes it the simplest described genome in grasses. The plant itself is small and easy to handle due to its undemanding growth requirements with a 15-week seed-to-seed lifecycle. At present, there are more than 40 ecotypes identified with varying responses to pathogen attack<sup>14,22</sup>. International resources for *Brachypodium* have been developed to provide seed stocks and genomics resources (<http://www.aber.ac.uk/plantpathol/brachyomics.htm>; <http://www.brachypodium.org/>).

### **Overall considerations**

*M. grisea* is clearly emerging as one of the most important model systems representing filamentous fungal pathogens of plants. Methods to explore the early phases (biotrophic) of rice blast establishment in a model host are likely to be extremely valuable to study fundamental aspects of fungal pathogenicity that are common to many diseases. An important consideration in any experiments involving studies between plants and their pathogens is the generation of high-quality inoculants. As the success of any infection is not evident until several days after inoculation, measures have to be in place to ensure and confirm the efficiency of infection before appearance of visible symptoms. Great care has to be taken with regard to the maintenance of the fungal stocks to maintain the virulence of the rice blast spores. Indeed, a key factor in the successful production of reproducibly infected host plants is the availability of a fungal spore population with both good virulence and high pre-determined germination efficiency. The procedures we describe for pre-checking inoculants involve simple light microscopic checks on the synchronous development of early infection structures in spores germinated *in vitro* that should be carried out for each batch of spores prepared for inoculation. Monitoring subsequent disease progression in pathogen challenged plants is much more difficult; we suggest that rice blast infection sites should be visible (and quantifiable) under UV illumination

after 72 h, at which stage a decision can be made whether to harvest or abandon the experiment. An alternative method for examining disease establishment at earlier phases is possible, using a variety of staining and high-resolution microscopy techniques<sup>31,32</sup>. Although such methods are useful for gaining experience in handling the disease, all take several hours to complete, they are destructive and cannot be carried out rapidly to accommodate routine sampling demands when monitoring dynamic changes.

Our experience has shown that 150 plants planted in several trays will provide sufficient material for efficient extraction of metabolites and subsequent metabolomic studies (see accompanying *Nature Protocol* by Beckmann *et al.*<sup>33</sup> describing in detail flow injection electrospray–mass spectrometry (FIE-MS) fingerprinting). The present method is thus based on handling this experimental batch size to ensure that all harvesting can be carried out in a window of 30 min in the middle of the light period. There is good evidence that the physiology of an individual leaf will vary depending on its developmental status when harvested. Therefore, for effective data alignment and informative data mining, it is important that samples are taken only from leaves at the same developmental stage from plants grown under identical conditions. As interactions between fungal pathogens and their hosts are complex and dynamic, an important aspect of the overall experimental strategy is to confirm that different batches of infected plants have behaved in the same way. One important aspect of the procedure therefore is to demonstrate how to check that data can be aligned when generated using different batches of infected plants.

This protocol describes the use of metabolome fingerprinting analysis for a high-throughput, first-pass evaluation of each sample class representing either different phases of the colonization process or a different batch of infected material. Unlike transcriptomics and proteomics experiments, where signal detection can be linked and constrained to the host or pathogen gene products, the same metabolite derived from either host or pathogen will have an identical chemical structure. The genomes of many pathogenic fungal species have not yet been fully sequenced and so in these circumstances we suggest that metabolome analysis should yield informative ‘omics’ level data in any host pathogen system. This protocol is ideal for a preliminary assessment of interacting metabolome changes in both host and pathogen associated with the diseased state to highlight areas of metabolism for deeper analysis in the future. The sampling rationale, data alignment and data mining strategies (described in detail in accompanying *Nature Protocol* by Enot *et al.*<sup>34</sup>) are also appropriate for proteomic and transcriptomic level studies. Indeed, we advocate that high-throughput analysis by metabolomic fingerprinting provides an efficient tool for investigating sample characteristics prior to investment in more expensive studies.

## **MATERIALS**

### **REAGENTS**

- Seeds of *B. distachyon* can be obtained from Brachyomics (University of Wales Aberystwyth UK. <http://www.aber.ac.uk/plantpathol/brachyomics.htm>). *B. distachyon* ecotype ABR 1 originated from Daman (Turkey) and undergoes a susceptible interaction with rice blast strain Guy11
- *M. grisea* (anamorph: *Pyricularia oryzae*) (T.T. Hebert) M.E. Barr strain Guy11 (mating type MAT1-2 from French Guiana) can be obtained from Prof. Nick Talbot (University of Exeter, UK)

- Water: Milli-Q quality ( $\leq 18 \text{ M}\Omega\cdot\text{cm}$ )
- Chloroform: GLC—Pesticide residue grade (Fisher, cat. no. C/4963/15)
- Methanol for extraction: trace analysis grade (Fisher, cat. no. M/4020/17)
- Autoclaved gelatine water (0.2% wt/vol)
- Liquid nitrogen (BOC)
- Levington’s Universal Compost (Levington Horticulture)
- 1,16-Hexadecanediol (Aldrich, cat. no. 177482-1G)
- Glucose (Fisher, cat. no. G/0500/61; a constituent of complete media agar (CMA))

## PROTOCOL

- Peptone (Difco, cat. no. 0118-17-0; a constituent of CMA)
- Yeast extract (Lab M, cat. no. 13295/134; a constituent of CMA)
- Casamino acids (Sigma, cat. no. A-2427; a constituent of CMA)
- Biotin (Sigma, cat. no. B4501; a constituent of CMA)
- Pyridoxine (Aldrich, cat. no. P9630-100MG; a constituent of CMA)
- Thiamine (Sigma, cat. no. T3902; a constituent of CMA)
- Riboflavin (Sigma, cat. no. R-4500; a constituent of CMA)
- p-Aminobenzoic acid (Melford, cat. no. A0601; a constituent of CMA)
- Nicotinic acid (Aldrich, cat. no. N4023-100G; a constituent of CMA)
- NaNO<sub>3</sub> (Fisher, cat. no. S/5560/53; a constituent of CMA)
- KCl (Fisher, cat. no. P/4280/53; a constituent of CMA)
- MgSO<sub>4</sub> (Fisher, cat. no. M/1050/53; a constituent of CMA)
- KH<sub>2</sub>PO<sub>4</sub> (Fisher, cat. no. P/4800/6; a constituent of CMA)
- Agar (Duchefa Biochemie, cat. no. M1002.0500; a constituent of CMA)

### EQUIPMENT

- Cooled walk-in growth room for plants (e.g., Polysec, R. J. Hicks Refrigeration)
- Gallenkamp Illuminated Cooled Incubator 9
- Hemocytometer (Hawksley)
- Artist airbrush (Humbrol)
- Polythene bags (610 × 816 mm)
- Leaf punch or segment cutter
- Stainless-steel balls (4 mm)
- Miracloth
- Filter paper (Whatman International, 85 mm)
- Sterile Petri dishes (9 cm)
- Single-edged razor blade
- Microbiological glass spreader
- Mounted needle
- Parafilm
- Gelbond (Cambrex, cat. no. 53734)
- Microvials: 2-ml 'safe lock' (Eppendorf)
- Oak Ridge centrifuge tube, PPCO, size 50 ml
- 5-mm cork borer
- Domestos bleach (Unilever)
- Aniline blue (George T. Gurr, cat. no. 2642), optional
- 1 M KOH (if staining with aniline blue)

- Sterile distilled water
- Glass slides (if staining with aniline blue)
- 0.067 M K<sub>2</sub>HPO<sub>4</sub> (if staining with aniline blue)

### REAGENT SETUP

**CMA medium** To make 1 l of CMA, dissolve the following in dH<sub>2</sub>O: 50 ml nitrate salts (see below), 1 ml trace elements (see below), 10 g D-glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 1 ml vitamin solution (see below) and 15 g of agar per 1 l of media (pH to 6.5). Dispense 20 ml aliquots into 9-cm Petri dishes.

**Trace elements:** 80 ml dH<sub>2</sub>O, 2.2 g ZnSO<sub>4</sub> · 7H<sub>2</sub>O, 1.1 g H<sub>3</sub>BO<sub>3</sub>, 0.5 g MnCl<sub>2</sub> · 6H<sub>2</sub>O, 0.5 g FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.17 g CoCl<sub>2</sub> · 6H<sub>2</sub>O, 0.16 g CuSO<sub>4</sub> · 5H<sub>2</sub>O, 0.15 g Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O, 5 g Na<sub>4</sub>EDTA. This stock solution can be made up in advance and stored at 4 °C.

**Vitamin supplement:** Add the following compounds in order, boil and then let cool (brown precipitate will then settle down to bottom, remove supernatant and discard precipitate), 0.001 g l<sup>-1</sup> biotin, 0.001 g l<sup>-1</sup> pyridoxine, 0.001 g l<sup>-1</sup> thiamine, 0.001 g l<sup>-1</sup> riboflavin, 0.001 g l<sup>-1</sup> p-aminobenzoic acid and 0.001 g l<sup>-1</sup> nicotinic acid. This stock solution can be made up in advance and stored in a dark glass bottle at 4 °C.

**Nitrate salts:** 6 g l<sup>-1</sup> NaNO<sub>3</sub>, 0.5 g l<sup>-1</sup> KCl, 0.5 g l<sup>-1</sup> MgSO<sub>4</sub>, 1.5 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, make up to 1 l with dH<sub>2</sub>O. This stock solution can be made up in advance and stored at 4 °C.

**Extraction solvent for metabolome analysis** Ensure enough extraction mix is prepared for all samples of the experiment to minimize variability. Use a pre-cleaned (thoroughly rinsed with water, methanol and chloroform) screw cap glass bottle of appropriate size. Add solvents in the order: water–methanol–chloroform in the ratio 1:2.5:1 (vol:vol:vol). Close bottle, shake to mix thoroughly and store at -20 °C. For sample extraction, aliquot extraction mix into 50 ml screw cap glass bottle for convenience and avoid cross-contamination of extraction mix with samples during pipetting.

**Gelatine water** 0.2% (wt/vol) gelatine in de-ionized water should be autoclaved at 121 °C for 2 min and stored at 4 °C.

**Autoclaved compost** Compost should be autoclaved at 121 °C for 20 min.

**1% Sodium hypochlorite** Domestos (Unilever) bleach diluted in de-ionized water. This stock solution can be made up in advance and stored at 4 °C.

**Hexadecanediol** Make a stock solution of 1 mg/ml hexadecanediol in 100% ethanol and dilute 1/1000 in distilled water before use. This stock solution can be made up in advance and stored at 4 °C.

## PROCEDURE

### Preparation of long-term stocks of virulent fungal pathogen

1| Obtain a fungal inoculum that has been prechecked for genetic purity and virulence from a laboratory experienced in rice blast research.

■ **PAUSE POINT** For long-term storage and transport, it is common to supply spores of *M. grisea* in a desiccated state after allowing mycelia to grow through filter-paper disks placed on top of solidified growth medium in a sterile Petri dish.

2| Prepare CMA plates with 10 × 1 cm sterile squares of Whatmann 3-MM filter paper arranged on the surface. Inoculate center of dish with a square of filter paper containing desiccated fungal hyphae from stocks.

3| Incubate plate for 10–12 d in a controlled temperature room at 26 °C with a 16-h light and dark cycle. After this time, fungal hyphae will have grown through the medium and into the filter-paper disks (see Fig. 2).

4| Transfer filter paper disks aseptically into new 9-cm Petri dishes, place into a desiccator containing silica gel and allow to dry for 4 d before sealing with Parafilm and storing at -20 °C. While the filter papers are drying, proceed with Step 5.

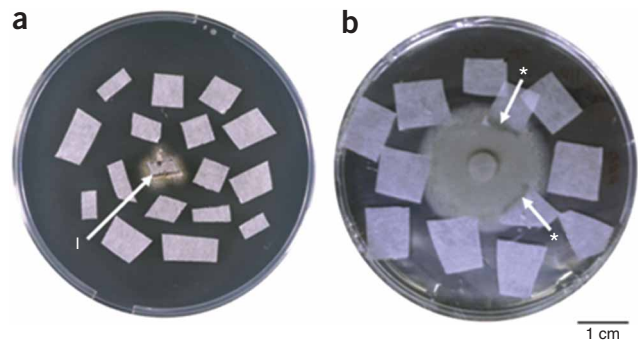
■ **PAUSE POINT** Filters can be stored at -20 °C.

5| Remove a number of agar plugs containing fungal tissue from the stock plate using a sterile 5-mm cork borer and inoculate onto several CMA plates containing 10 × 1 cm sterile disks. Repeat Steps 3 and 4 until 50–100 filter paper disks are produced. These disks now provide a stock of fresh inoculum.

### Preparation of fungal inoculants for challenge of host plants

6| Remove a filter-paper disk containing desiccated fungal spores from storage containers aseptically and inoculate onto a plate of CMA. Incubate plate for 10 d in a controlled temperature room at 26 °C with a 16-h light and dark cycle. After this time, fungal growth should cover the surface of the medium.

**Figure 2** | Preparation of long-term stocks of virulent *Magnaporthe grisea*. (a) Fungal inoculum (I) is placed in the center of a Petri dish containing grown media overlain with filter-paper squares, cultures are grown at 26 °C with a 16-h light and dark cycle for conidia harvesting. (b) Six days after inoculation demonstrating fungal overgrowth and sporulation onto filter-paper squares (\*) before harvesting and preservation of filter paper for fungal stocks at 10–12 d after inoculation.



7| Remove a number of agar plugs containing fungal tissue from the stock plate using a sterile 5-mm cork borer and inoculate onto CMA (Fig. 3a). To generate sufficient conidia for inoculation of 5 trays (15" × 19") containing ~150 plants, it is recommended that ~10 dishes are inoculated.

8| Incubate plates for 10 d in a controlled temperature room at 26 °C with a 16-h light and dark cycle. After this time, fungal hyphae will have spread through the dish and aerial hyphae with mature conidia should now cover the surface of the medium (see Fig. 3b). Discard any plates with obvious contaminants or with non-uniform growth.

9| Select two or three plates at random and harvest conidia by gently washing the surface of each with 3 ml of sterile distilled water.

▲ **CRITICAL STEP** Do not use conidia from fungal plates > 15 days old.

10| Filter the resulting suspensions through sterile Mira cloth and place into an Oak Ridge Centrifuge tube, PPCO, size 50 ml.

11| Centrifuge tubes at 5,000g for 10 min at room temperature (18–23 °C).

12| Re-suspend pellet in distilled water containing hexadecanediol to a final concentration of  $5 \times 10^5$  conidia ml<sup>-1</sup>. As a guide, the washed conidia from a single 9-cm Petri dish can be re-suspended in 3 ml; spore density should be checked under a hemocytometer before dilution.

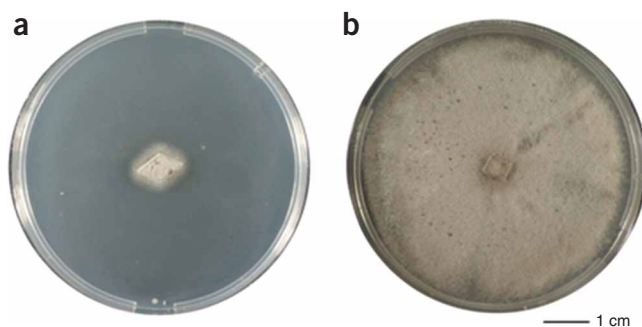
13| Spot 1 ml of the conidial suspension at regular intervals (4 across and 4 down) across a 12-cm square Petri dish containing Gelbond (hydrophobic side up) and examine under a light microscope at regular time intervals (e.g., 0, 4, 8, 12, 16 and 24 h) to assess the frequency of spore germination, germ tube extension and hooking after 4–8 h and appressorium elaboration after 24 h (Fig. 1 illustrates these developmental stages). Discard any fungal cultures that fail to show >75% germination after 8 h or 75% appressorium production after 24 h.

14| Harvest conidia to provide an inoculum for challenging host plants within 24–48 h of the germination viability check. If desired, large-scale *in vitro* germination of conidia on 'gel bond' can be carried out, as described in Box 1.

▲ **CRITICAL STEP** Ensure that fungal spores are checked for germination efficiency 1 day before harvesting for plant inoculation (Steps 9–13).

### Growth of host plants for challenge with *M. grisea*

15| To prepare *B. distachyon* plants for infection, follow option A. To prepare *Hordeum vulgare* or *Oryza sativa*, follow options B or C, respectively. The anticipated results describe the use of the model system *B. distachyon* which is smaller and easier to handle than the two crop species.



**Figure 3** | CMA plates of *Magnaporthe grisea* Guy11 cultures grown at 26 °C with a 16-h light and dark cycle for conidia harvesting. (a) One day after inoculation; (b) 10 d after inoculation.

#### (A) Preparation of *B. distachyon* for challenge with *M. grisea*

- (i) Fill up a 5 × 4 strip of 4 inch square 'Vacupots' (or equivalent) with sterilized Levington's Universal Compost supplemented with gravel (50:50; vol/vol) to improve drainage and place in a 15 inch × 19 inch seed tray (or equivalent).
- (ii) Sow *B. distachyon* seeds (9 seeds/pot) into compost, water and grow for 21–28 d in an environmentally controlled (and preferably sealed) growth room (e.g., Polysec) under a 16-h light period at 23 °C (± 2 °C). **Figure 4a** illustrates a typical tray of *B. distachyon* seedlings at the correct stage for inoculation.
- (iii) Proceed directly to Step 16.

#### (B) Preparation of *H. vulgare* for challenge with *M. grisea*

- (i) Fill up a 15 inch × 19 inch seed tray with sterilized Levington's Universal Compost.

**BOX 1 | LARGE-SCALE *IN VITRO* GERMINATION OF CONIDIA ON ‘GEL BOND’**

1. Prepare 20 plates of CMA inoculated with Guy11 for each desired time point and incubate for 13 d as described in Steps 15–16.
2. Harvest all plates with a glass spreader and distilled water and filter the resulting suspensions through sterile Miracloth and place into an Oak Ridge Centrifuge tube, PPCO, size 50 mL.
3. Centrifuge tubes at 5000g for 10 min at room temperature.
4. Re-suspend pellet in distilled water containing 1,16-hexadecanediol to a final concentration of  $5 \times 10^5$  conidia  $\text{ml}^{-1}$ . Hexadecanediol is a plant cutin monomer that induces appressorium differentiation in *Magnaporthe grisea*.
5. Spot 1 ml of the conidial suspension at regular intervals (4 across and 4 down) across a 12-cm square Petri dish containing Gelbond (hydrophobic side up). Germinating conidia will adhere tightly to Gelbond.
6. Remove excess water at the desired developmental stage (e.g., 4, 8, 12, 16 and 24 h) and harvest adhered fungal material by scraping Gelbond surface using a razor blade and into an Eppendorf tube. All samples should be rapidly snap-frozen in liquid  $\text{N}_2$  and kept at  $-80^\circ\text{C}$ .

(ii) Sow *H. vulgare* seeds (30–40 seeds per tray) into compost, water well each day and grow for 10–15 d in an environmentally controlled (and preferably sealed) growth room under a 16-h light period at  $23^\circ\text{C} (\pm 2^\circ\text{C})$

(iii) Proceed directly to Step 16.

**(C) Preparation of *O. sativa* for challenge with *M. grisea***

(i) Fill up a 15 inch  $\times$  19 inch seed tray with sterilized Levington’s Universal Compost.

(ii) Sow *O. sativa* seeds (32 seeds per tray in 4 rows of 8) into compost, water well each day and grow for 21–30 d (to 3–4-leaf stage) in an environmentally controlled (and preferably sealed) growth room under a 10-h light period at  $27^\circ\text{C} (\pm 2^\circ\text{C})$ .

(iii) Proceed directly to Step 16.

**▲ CRITICAL STEP** To reduce potential ‘block effects’ related to position in the growth room, it is good practice to randomize the position of plant trays each morning before watering a week before infection and during infection.

**16|** Harvest germination-tested conidia using 5 ml of sterile gelatine water per plate from  $10 \times 10$ -day-old cultures of *M. grisea* grown on CMA as outlined in Steps 6–8.

**17|** Filter resulting suspension through sterile Miracloth and adjust to a final concentration of  $1 \times 10^5$  conidia  $\text{ml}^{-1}$ . A volume of  $\sim 50$  ml will be sufficient for the inoculation of five trays of plant material.

**Challenge of susceptible plants with *M. grisea***

**18|** To challenge *B. distachyon* plants with *M. grisea*, follow option A. To challenge *H. vulgare* or *O. sativa* with *M. grisea*, follow options B or C, respectively.

**(A) Challenge of *B. distachyon* with *M. grisea***

(i) Spray-inoculate *B. distachyon* plants at the five-leaf stage (21–28 d after sowing) with the conidial suspension using an artist’s airbrush.

(ii) Water plants well after spray-inoculation, cover with polythene bags for 48 h (**Fig. 4b**) and incubate in a sealed growth room under a 16-h light period at  $23^\circ\text{C} (\pm 2^\circ\text{C})$ .

(iii) Remove bags and grow for a further 2–3 d in a controlled environment chamber at  $23^\circ\text{C}$  with a 16-h light/dark cycle.

(iv) Proceed directly to Step 19.

**(B) Challenge of *H. vulgare* with *M. grisea***

(i) Spray-inoculate *H. vulgare* plants at the three-leaf stage (10–15 d after sowing) with the conidial suspension using an artist’s airbrush.

(ii) Water plants well after spray-inoculation, cover with polythene bags for 48 h and incubate in a sealed growth room under a 16-h light period at  $23^\circ\text{C} (\pm 2^\circ\text{C})$ .

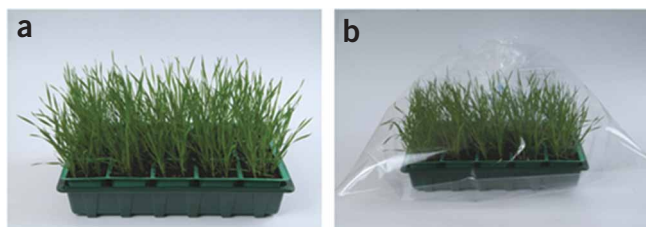
(iii) Remove bags and grow for a further 2–3 d in a controlled environment chamber at  $23^\circ\text{C}$  with a 16-h light/dark cycle.

(iv) Proceed directly to Step 19.

**(C) Challenge of *O. sativa* with *M. grisea***

(i) Spray-inoculate *O. sativa* plants at the three-leaf stage (30–35 d after sowing) with the conidial suspension using an artist’s airbrush.

(ii) Water plants well after spray-inoculation, cover with polythene bags for 48 h and incubate in a sealed growth room under a 10-h light period at  $27^\circ\text{C} (\pm 2^\circ\text{C})$ .



**Figure 4 |** *Brachypodium distachyon* ABR1 seedlings grown for 21–28 d in an environmentally controlled growth room under a 16-h light period at  $23^\circ\text{C} (\pm 2^\circ\text{C})$ . (a) A typical tray of *B. distachyon* seedlings at the correct stage for inoculation; (b) after spray-inoculation, cover with polythene bags for 48 h at previous growth conditions.

(iii) Remove bags and grow for a further 2–3 d in a controlled environment chamber at 27 °C with a 10-h light/dark cycle.

(iv) Proceed directly to Step 19.

**▲ CRITICAL STEP** Plants should always be inoculated at the same time of day for all replicate experiments to maintain the exact time difference between the first and consecutive harvests for all plants and experiments. The infection should be timed to be exactly in the middle of a photoperiod (e.g., if growth room lights turn on at 3 AM and off at 7 PM, then the infection and harvest time should be 11 AM). To ensure infection reproducibility, all plant trays should be sprayed with exactly 10 ml of inoculum using the same spray pattern.

**19|** The infected and control plant material can be misted every morning using a fine water spray to simulate morning dew. This has been shown to encourage rapid fungal growth and sporulation. For harvest within the 48-h bagging period, the tray should be removed from the polythene bag 2–3 h before the sampling to ensure that any effects from handling or changes in environment have dissipated.

**▲ CRITICAL STEP** Do not allow plants to dry out.

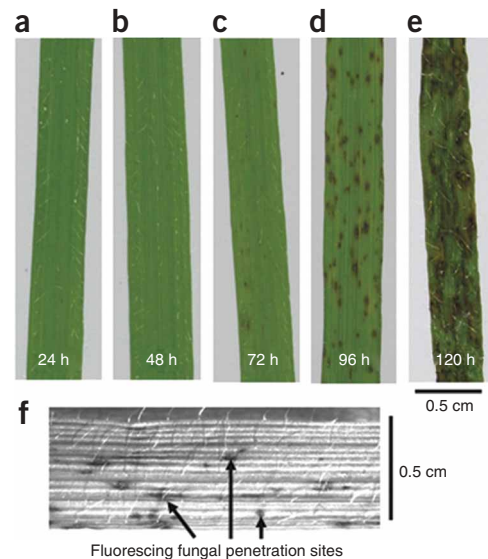
#### Assessment of disease progression

**20|** If desired, disease progression in pathogen challenged plants can be monitored at 72 h by quantifying lesion production, using a UV transilluminator. At this stage, a decision can be made whether to harvest or abandon the experiment. A good experiment should have 10–20 infection sites per 1 cm of leaf (see Fig. 5).

**21|** Score disease progression and reproducibility across all experiments at 96 h after inoculation using the 0–5 scale according to Valent and Chumley<sup>27</sup>. Six lesion types have been defined as follows for rice seedlings inoculated under conditions described. Type 0, no visible evidence of infection; type 1, uniform dark brown pinpoint lesions without visible centers; type 2, small lesions with distinct tan centers surrounded by a darker brown margin; type 3, small eyespot lesions ~2 mm long with tan centers surrounded by dark brown margins; type 4, intermediate size eyespot lesions, ~3–4 mm long; type 5, large eyespot lesions (~5 mm long for rice strain C039). Types 0 and 1 are considered nonpathogenic, or avirulent, interactions. Types 2, 3, 4 and 5 were considered pathogenic, or virulent, because conidia were produced from such infected tissues under high humidity conditions. Any experiment failing to produce type three to four lesions (shown in Fig. 5d,e) with an even covering of infection sites across the leaf should be discarded.

**▲ CRITICAL STEP** Check infection efficiency before using experimental material.

**22|** If required, lesion production can also be quantified using high-resolution microscopy after staining for fungal tissues (Box 2). Aniline blue staining is a popular method based on the protocol developed by Hood and Shew (1996)<sup>32</sup>. Confirmation of pathogenicity and maintenance of pathogenicity can be carried out as described in Box 3; see also Figure 6. Alternatively, proceed directly to Step 23.



**Figure 5 |** Development of disease symptoms on *Brachypodium distachyon* ABR1 leaves after infection with *Magnaporthe grisea* Guy11. (a–e) Over the protocol time-course; (f) lesion production can be quantified by examining for autofluorescent infection sites after 72 h or staining for fungal tissues using aniline blue. Any experiment failing to produce type 3–4 lesions (d and e) with an even covering of infection sites across the leaf should be discarded.

### BOX 2 | ANILINE BLUE STAINING TO ASSESS LEVEL OF FUNGAL TISSUE IN PLANT LEAVES

1. Cut infected leaf into 4-cm length and autoclave at 121 °C in 1 M KOH for 2 min.
2. Rinse three times in sterile distilled water and mount on glass slides in several drops of freshly prepared 0.05% water-soluble aniline blue dye in 0.067 M K<sub>2</sub>HPO<sub>4</sub>, pH 9.

#### Harvesting diseased leaf tissue

**23|** Examine individual plants and identify the youngest fully expanded leaf as these are normally more susceptible to infection than older leaves. In grasses, leaves are numbered according to the order of emergence with leaf 1 being basal (the oldest leaf) and leaf 5 at time of spraying being apical and the youngest leaf. The harvested leaf is the last leaf judged to have been fully emerged at the time of spraying (Fig. 7a). It is critical to harvest material from identically positioned leaves from all plants.

**▲ CRITICAL STEP** It is important that the correct leaves are selected for harvest.

### BOX 3 | CONFIRMATION OF PATHOGEN FROM LESIONS AND MAINTAINING PATHOGENICITY OF *MAGNAPORTHE GRISEA* STOCKS

1. Excise fresh material from infected plant parts (e.g., leaf, stem or sheath) and cut into small pieces around the area showing the blast symptoms, including the edge of the lesion (Fig. 6a).
2. Place all samples into a 9-cm Petri dish in which all surface sterilization and washings are performed.
3. Surface sterilize infected leaf samples with 1% sodium hypochlorite (bleach) for 30 min followed by 3 washes with sterile distilled water.
4. Place plant pieces in 9-cm Petri dishes lined with moist 3-MM filter paper and incubate at 25 °C (16-h light and dark cycle) for 24 h to encourage sporulation (Fig. 6b).
5. Examine leaf section under a stereo-dissecting microscope; abundant *M. grisea* growth and sporulation should be visible from and around the lesions which are gray, dense and bushy in appearance (Fig. 6c).
6. Pick up some conidia by brushing the needle across the sporulating lesion using a sterile moistened mounted needle. Place conidia onto CMA and incubate at 25 °C for 7–10 d in 16-h darkness and 8-h light.
7. Verify identity of *M. grisea* by checking the conidia morphology under a light microscope; mature conidia are three-celled, pyriform, and bear a basal appendage at the point of attachment to the conidiophore (Fig. 6d).

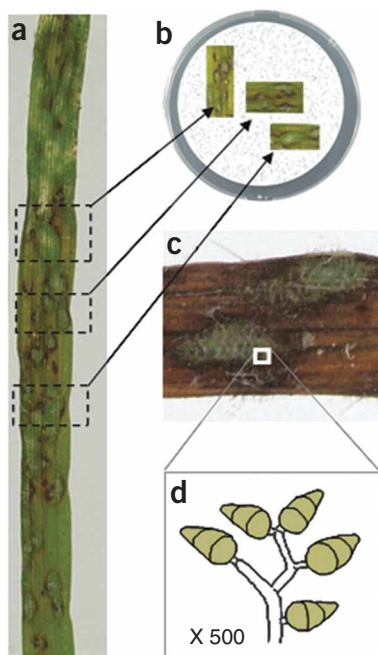
24| To sample pathogen challenged *B. distachyon* or *O. sativa* leaves, follow option A. To sample pathogen challenged *H. vulgare* leaves, follow option B. The protocol differs for barley as the susceptible seedlings are much larger than the other two grass species.

**(A) Sampling from *B. distachyon* or *O. sativa* leaves challenged by *M. grisea***

- (i) Harvest infected material by cutting the plant at the base of the stem using surgical scissors to ensure that it is not harvested a second time. The required leaf is excised at the base and a central 4-cm leaf blade section removed as shown in Figure 7a.
- (ii) Proceed directly to Step 25.

**(B) Sampling from *H. vulgare* leaves challenged by *M. grisea***

- (i) Harvest infected material by cutting the plant at the base of the stem using surgical scissors to ensure that it is not harvested a second time. The required leaf is excised at the base and a central 10-cm section removed.
- (ii) Using a hand-held punch (5-mm diameter), three-leaf disks are taken from the bottom, middle and top of the excised leaf section (Fig. 7b).
- (iii) Proceed directly to Step 25.



**Figure 6** | Confirmation of pathogen from lesions and maintaining pathogenicity of *Magnaporthe grisea* stocks. (a) Samples are removed from leaves displaying infection symptoms; (b) leaf sections are sealed in Petri dishes containing moistened filter paper; (c) lesions are inspected for fungal outgrowth; (d) fungal identity confirmed using micromorphology.

25| Place the leaf samples quickly into a 2-ml Eppendorf ‘Safelock’ tube containing a stainless-steel ball (for future extraction), snap-freeze in liquid N<sub>2</sub> and store at –80 °C.

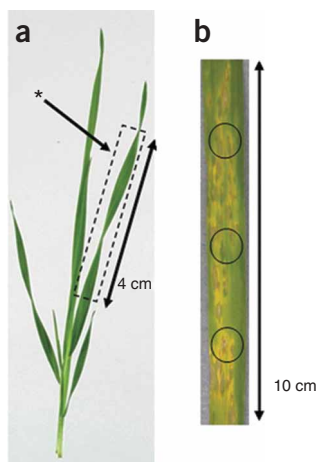
**! CAUTION** Work with liquid nitrogen only in well-ventilated areas; use PPE.

**■ PAUSE POINT** Samples can be stored at ultra low temperature (–80 °C) for up to 8 weeks before analysis.

**ANTICIPATED RESULTS**

The major objective of this protocol is to describe how to generate biological materials that would be suitable for the study of dynamic interactions between an invading fungal pathogen and its host plant. A timeline outlining consecutive sections of this protocol is presented in **Table 2**. The procedure is scalable to allow generation of sufficient samples for microscopy, cell biology, biochemical and gene expression studies at the level of transcriptome, proteome and metabolome. The early stages of the pathogen invasion process before the appearance of visible lesions are difficult to monitor to confirm the synchronicity of infection between plants and reproducibility between experimental batches. In the anticipated results, we describe the validation of infection success and sampling strategy by testing for similarity of sample classes (in this case an infected leaf time series) using metabolite fingerprinting (FIE-MS) and linear discriminant

**Figure 7 |** *Brachypodium distachyon* (ABR 1) at five-leaf stage indicating harvest leaf and sampling zone. (a) The last leaf judged to have been fully emerged at the time of spraying (\*) is selected for harvest, to harvest, the leaf is excised at the base and a central 4-cm section removed; (b) excised 10 cm *Hordeum vulgare* leaf section indicating points of leaf disk extraction.



**TABLE 2 |** Timeline outlining consecutive stages of protocol for challenging *Brachypodium distachyon* with *Magnaporthe grisea*.

Major events	Approximate time	Steps
Creating fungal stocks	1 month	1–5
Growing fungal plates for inoculation	10–14 d	6–8
Preparing fungal inoculant	1 h	16–17
Ensuring spore germination efficiency	24 h	9–13
Growing <i>Brachypodium distachyon</i>	21–28 d	15
Infecting host	10–20 min	18
Disease development	6 d	19
Harvesting infected material	Dependent on number of samples harvested	23–25

analysis (LDA). Both of these procedures are described in detail in accompanying *Nature Protocols* by Beckmann *et al.*<sup>33</sup> and Enot *et al.*<sup>34</sup> and the experimental steps are summarized in **Box 4**.

### Assessment of disease progression

Depending upon the experimental requirements infected and control tissues can be harvested at any time during the infection period of this protocol. The typical appearance of disease symptoms in *B. distachyon* seedlings infected with a compatible rice blast strain, over the protocol time-course, is illustrated with close up photographs of individual leaves in **Figure 5a–e**. From our experience working with *M. grisea* in compatible infections using *B. distachyon*, *H. vulgare* and *O. sativa* (D.P., unpublished results), visible symptoms of the disease are first observed 72–96 h after infection (spraying)<sup>14</sup>. The general strategy employed to assess disease progression is to sample tissues when required throughout the protocol time course (as outlined in Steps 20–22) and wait until 96 h have elapsed before evaluating each individual experiment for disease efficiency. If attenuated disease symptoms are observed after 96 h, the experiment should be discarded and repeated with conidial stocks that have been confirmed with regard to pathogenicity (refer to **Box 3**).

### Reproducible harvesting

During disease progression, infected leaves become gradually more desiccated, altering the total leaf wet weight but not total biomass. For example after 72–96 h of infection with *M. grisea*, the susceptible ecotypes ABR 1 and 7 can show up to a 10% reduction of water in the leaf tissue. By 120 h, this can rise to 50–70% water loss in susceptible plants. At this stage of the infection, nearly twice the area of infected leaf material is required to equal the same fresh weight of healthy material (**Fig. 8**). To overcome this problem, measured leaf sections, i.e., same length of mid-leaf segment, are harvested to ensure that equal biomass is harvested regardless of treatment. In addition, all harvesting should take place inside the growth room to reduce any further stress responses caused by transporting plants.

## BOX 4 | USE OF METABOLITE FINGERPRINTING AND LINEAR DISCRIMINANT ANALYSIS TO VALIDATE REPRODUCIBILITY OF PATHOGEN INFECTION AND SAMPLING PROCEDURE

1. Extract metabolites from each leaf section (or three leaf punches) by grinding in a ball mill in 1 ml of solvent comprising water and methanol for polar metabolites and chloroform for more lipophilic compounds at a ratio of 1:2.5:1 (H<sub>2</sub>O:CH<sub>4</sub>O:CHCl<sub>3</sub>) and subject extract to metabolite fingerprinting by flow injection electrospray mass spectrometry (FIE-MS). Follow the procedure described in detail in the accompanying *Nature Protocol* by Beckmann *et al.*<sup>33</sup>.

**! CAUTION** Halogenic reagents and solutions should be disposed with halogenic waste.

2. Perform principal component linear discriminant analysis (PC-LDA, or an alternative such as partial least squares regression discriminant analysis) to examine both consistency of samples from each class (time points) and reproducibility between different batches of infected plants. Guidelines are given in ANTICIPATED RESULTS text and details are presented in the accompanying *Nature Protocol* by Enot *et al.*<sup>34</sup>.

3. Check that each sample class shows clear grouping and is reasonably well discriminated from others (see **Fig. 9**). Good discrimination between classes could indicate potentially good prospects for data modeling even if biological variance is relatively high.

4. Calculate associated class separation metrics (in the case of PC-LDA use eigenvalues) and classification accuracy from the confusion matrix (see **Table 3**) and make a decision whether to continue with the experimental material for further in-depth studies. As a general rule, eigenvalues < 2.0 in models comparing two sample classes indicate poor class discrimination.

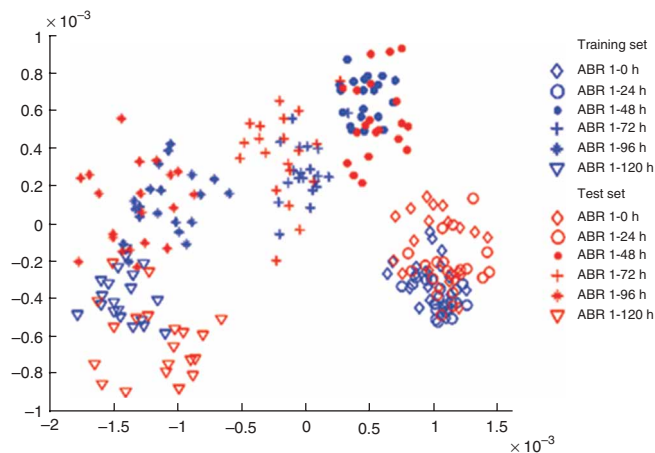
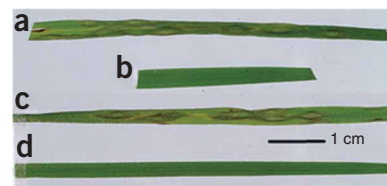
**Infection batch validation by metabolite fingerprinting**

Comparison of FIE-MS metabolome fingerprints can be used to demonstrate the reproducibility of the plant infection protocols. Validation of *in planta* infection was carried out using a susceptible ecotype of *B. distachyon* (ABR 1) infected with Guy11 in two separate experiments conducted several months apart. Samples from each

experiment were harvested at 0, 24, 48, 72, 96 and 120 h after inoculation and analyzed by FIE-MS (see accompanying *Nature Protocol* by Beckmann *et al.*<sup>33</sup>) followed by principal component analysis (PCA) and LDA (see **Box 4**). PC-LDA results are visualized in **Figure 9**. One experimental batch of infected plants was used to generate a set of training data (blue symbols) whereas the other batch (grown several weeks later) was used as test data (red symbols). The accompanying *Nature Protocol* by Enot *et al.*<sup>34</sup> provides a detailed discussion of concepts and procedures for sample classification. Test and training sets are used to cross-validate the LDA to assess the reliability and generalizability of the findings. From **Figure 9**, test and training data overlaid each other well (high classification accuracy of test-set) and sample classes show good discrimination. The “leave-one-out” confusion matrix presented as **Table 3** shows that the metabolomes of each sample class show no misclassification with one another. In addition to this, examination of other model measures (such as eigenvalue) permit an assessment of robustness of the metabolome model and an indication of how interpretable it is likely to be in regards to highlighting metabolites that are biologically significant between sample classes (see accompanying *Nature Protocol* by Enot *et al.*<sup>34</sup>). From the information provided in **Table 3**, the protocol can be interpreted as producing samples having similar metabolomic content within each sample class and with little misclassification between sample classes; confirming the robustness and reproducibility of the infection protocol for this complex and highly dynamic system.

An alternative procedure not mentioned in this protocol that can be applied to normalize samples is to pool infected leaves representing each time point, freeze dry tissues, and then weigh out accurately equal amounts of material sufficient for each extraction. However large, temporally dynamic changes in metabolism occur in host-pathogen interactions that have to be taken into consideration. These changes can differ between individual leaves, actual spore inoculation density, success of germination and other factors relating to individual leaf physiology. Therefore, under such circumstances, a pool of infected leaf samples could contain individual leaf sections in which disease progression was either less advanced or more advanced than in the majority of samples. Homogenizing material would clearly only lead to a reduction in the distinctiveness of individual sample classes. Both of these factors can have a confounding effect on subsequent data analysis. The sampling procedure we recommend retains each excised leaf section as an individual sample thus preserving the natural range of biological variability in metabolite levels to determine how similar samples are within an individual class (in this case, time series). A sufficiently high number of biological replicates then allows for the removal of any major outlier detected in e.g., PCA without confounding further in-depth data analysis.

**Figure 8** | During disease progression, infected *Brachypodium distachyon* leaves become gradually more desiccated, altering the leaf wet weight but not biomass. (a) Ninety-six hours after infection with *Magnaporthe grisea* (sample weight = 0.05 g); (b) control (sample weight = 0.05 g); (c) 96 h after infection with *M. grisea* (sample length = 4 cm); (d) control (sample length = 4 cm). Nearly twice the area of infected leaf material is required to equal the same fresh weight of healthy material (a,b). To overcome this problem, measured leaf sections are harvested to ensure that equal biomass is harvested regardless of treatment (c,d).



**Figure 9** | Linear discriminant analysis (LDA) score plots of flow injection electrospray mass spectrometry (FIE-MS) fingerprints representing *Brachypodium distachyon* ABR 1 challenged with *Magnaporthe grisea* Guy11 from two separate experiments (training set represented by blue symbols and test set by red symbols).

**TABLE 3** | Linear discriminant analysis metrics (classification accuracies and eigenvalues from **Fig. 9**).

	Class prediction positive ion				
	1	2	3	4	5
True Sample Class					
<b>1</b>	20	0	0	0	0
<b>2</b>	0	20	0	0	0
<b>3</b>	0	0	20	0	0
<b>4</b>	0	0	0	20	0
<b>5</b>	0	0	0	0	20
DF	Eigenvalue				
1	23.5180				
2	4.3940				
3	3.9810				
4	1.6980				
5	1.5000				

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