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In Vitro Induction of Infection-Related Hyphal Structures in Plant Pathogenic Fungi

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Abstract

In recent years, a voluminous amount of genomic data has been generated for several plant pathogenic fungi. Multiple studies have utilized these genomic data to advance our knowledge about the molecular mechanisms of plant pathogenesis. However, not all plant pathogenic fungi share the same infection strategies, and several genes have been identified that are crucial for plant pathogenesis in one fungus, but dispensable in others. In order for data on biological relevance to keep pace with accumulating genomic data, new biological assays need to be developed for several pathogenic fungi. Accordingly, we have developed an in vitro assay that allows us to monitor morphological changes in hyphal development as the head blight pathogen *Fusarium graminearum* infects wheat. Using previously frozen detached wheat glumes, we are able to monitor both subcuticular and intercellular hyphal development of *F. graminearum*. The method described takes only 3–5 days from inoculation to microscopic observation (depending on time point) and does not require any elaborate laboratory equipment or supplies. This method could be adapted for different necrotrophic or hemibiotrophic pathogens, on their host tissue types, in order to characterize their hyphal differentiation in vitro.

Keywords: *Fusarium* head blight, *Gibberella zeae*, glume, infection hyphae, subcuticular hyphae

1. Introduction

Plant pathogenic fungi differentiate morphologically distinct hyphal structures at various times during infection. For example, the spores of the rice blast fungus, *Magnaporthe oryzae*, germinate and eventually differentiate a dome-shaped appressorium that mediates penetration of the rice leaf epidermis (1). The ability to induce *M. oryzae* appressoria in vitro has advanced the understanding of the physiological and genetic cues required for appressoria formation (2–4). A similar in vitro assay in other plant pathogenic fungi, coupled with the increase of readily available genomic data, will help elucidate the genetic requirements of infection-related development.

Fusarium graminearum can cause a myriad of plant diseases, though it is most notorious for causing fusarium head blight of wheat and barley (5,6). Many of the molecular requirements of pathogenicity and virulence have been elucidated over the past decade; however, a simple in vitro assay for pathogenic hyphal development was lacking. Unlike plant pathogenic fungi such as *M. oryzae*, *F. graminearum* does not differentiate an appressorium before invading plant tissue. However, it does differentiate coral-like subcuticular hyphae and wide dikaryotic hyphae upon wheat and barley invasion (7–9). We have used the following protocol to induce these infection-related structures in vitro and to demonstrate that the *Δgpmk1* MAPK mutant is unable to induce wide bulbous infection hyphae (10).

2. Materials

2.1 Inoculation Material

1. Yeast Malt Extract Agar (YMA) plates: 4 g malt extract, 4 g sucrose, 4 g yeast extract, 15 g agar, to 1 L of deionized-distilled H₂O (ddH₂O).
2. Carboxymethyl cellulose (CMC) liquid media: 15 g CMC (low viscosity, sodium salt), 1 g yeast extract, 1 g NH₄NO₃, 1 g KH₂PO₄, 0.25 g MgSO₄...7H₂O.
3. Macroconidia of *Fusarium graminearum*: macroconidia can be generated in CMC media or on solid YMA plates (see Note 1).
4. 0.05% Tween 20 solution.
5. Frozen wheat heads: Collect wheat heads at anthesis from wheat variety “Norm.” Store the wheat heads in a plastic bag at –20°C (see Note 2).
6. Ultraviolet light for sanitizing wheat glumes.
7. 4% (w/v) water agar.

2.2 Components for Fixing Inoculated Glumes

1. 24-well cell culture plate.
2. Clearing solution A: 75 mL ethanol, 25 mL glacial acetic acid.
3. Clearing solution B: 50 mL ethanol, 10 mL glacial acetic acid, 10 mL glycerol.
4. Lactophenol blue staining solution: 13.5 mL lactic acid, 13.5 mL phenol, 12 mL ddH₂O, 1 mL 0.4% trypan blue solution.
5. Destain solution: 60 mL glycerol, 40 mL ddH₂O.

2.3 Components for Dissecting/Mounting Fixed Glumes

1. Dissecting microscope.
2. Surgical Scalpel.
3. Fine metal forceps.
4. Microscope slides and coverslips.
5. Bright-field compound microscope with camera (camera optional).

3. Methods

3.1 Growth of *F. graminearum* on Solid YMA or Liquid CMC Media to Produce Macroconidia

1. Inoculate YMA plates or 50 mL CMC media with 50 μ L of a 1×10^5 macroconidia suspension (see Note 1).
2. If using YMA plates to generate macroconidia, place the inoculated plates in an incubatory shaker set at 28°C. Incubate for 4–5 days.
3. If using liquid CMC cultures to generate macroconidia, place the 50 mL cultures in a rt 200 rpm and 25°C for 5 days.

3.2 Harvest the Macroconidia

1. If using YMA, add 1.5 mL of 0.05% tween to the plate and release macroconidia with a sterile-glass rod. Collect macroconidia suspension and filter through miracloth (Calbiochem) to remove hyphal debris.
2. If using CMC, filter the media/spore suspension through miracloth to remove hyphal debris.
3. Assess the macroconidia concentration with a hemacytometer and dilute to 1×10^6 in 0.05% tween.

3.3 Prepare Wheat Glumes for Inoculation

1. Remove wheat heads from -20°C . Carefully remove glumes from the florets and place on 4% water agar with the cavity (adaxial side) facing up (see Note 3; Figure 1).
2. Place water agar plates, without lids, under an ultraviolet light for 30–60 s to sanitize the glumes.

3.4 Inoculate and Incubate Detached Wheat Glumes

1. Collect 2000 L of macroconidia suspension (concentration between 10^4 and 10^6 macroconidia per mL; see Note 4) in a P200 micropipettor.
2. Aliquot approximately 20–30 μ L of the suspension onto the adaxial side of each glume (see Note 3; Figure 1).
3. Incubate the glumes at room temperature for 1 min, and then remove the macroconidia suspension from the glumes.
4. Incubate the wheat glumes at room temperature for given time points (see Note 5).

Figure 1. Wheat heads and florets. (a) Side view of wheat head showing rachis, florets, and area where a floret was detached (arrow). (b) Front view of a wheat head showing where floret was detached (arrow). (c) Detached floret showing the abaxial and adaxial side of wheat glumes.

3.5 Fixing and Mounting Wheat Glumes for Microscopy

1. After a given time point, remove glumes from water agar plate and place into well of 24-well cell culture plate containing 1 mL of clearing solution A (see Note 6). Incubate overnight (~16 h) at 75 rpm and room temperature.
2. Move the glumes to another well containing 1 mL clearing solution B. Incubate for 4 h at 75 rpm and room temperature.
3. Move the glumes to another well containing 1 mL of lactophenol blue staining solution (see Note 7). Incubate overnight (~16 h) at 75 rpm and room temperature.
4. Move glumes to another well containing 1 mL of 60% glycerol for 5 min to remove most of the residual lactophenol blue. After 5 min incubation, move to fresh 60% glycerol and incubate for at least 3 h.
5. With the aid of a dissecting scope, use the forceps and surgical scalpel to cut washed glumes into ~1 mm² sections for oblique views or 0.25 mm thick strips for cross-section views (see Note 8).
6. Move the sections to a microscope slide containing ~50 μ L 60% glycerol. Overlay with coverslip and examine under microscope (see Figure 2 for some expected results).

Figure 2. Some expected results. (a) Coral-like subcuticular hyphae after 24 h. (b) Bulbous infection hyphae after 48 h. (c) Cross-section view of glume inoculated on adaxial side (*arrow*). Note the invasion of lower mesophyll cells by *F. graminearum* hyphae. Hyphae in all panels stained with lactophenol trypan blue. Scale bar = 10 μ m in all panels.

4. Notes

1. Many laboratories use CMC to generate *F. graminearum* macroconidia, and it has become an accepted standard for the generation of macroconidia; we have also had good results using this method. However, we obtain 10–100-fold more macroconidia when using YMA, and some of the mutants generated in our laboratory do not sporulate well in CMC. Users are encouraged to try both methods and see which works better for their given scenario.
2. Subcuticular and bulbous invasion hyphae are more readily formed on previously frozen glumes compared to freshly detached wheat glumes (unpublished data).
3. We have inoculated macroconidia and ascospores on both the abaxial and adaxial side of wheat glumes (10). On the abaxial side, the hyphae appear to readily penetrate stomata, but the development of morphologically distinct structures is not as common as when the spores are inoculated onto the adaxial side of the glume.

4. When beginning our experiments, overinoculation often caused heavy invasion and staining that made distinguishing morphological structures difficult. We have since had success with macroconidia concentrations between $5 \times 10^4 \text{ mL}^{-1}$ and $1 \times 10^6 \text{ mL}^{-1}$. Before pursuing these experiments, we recommend users empirically test various macroconidia concentrations to see which yield suitable results.
5. Subcuticular hyphae and bulbous invasion hyphae are readily observed at the 24 h incubation time point when *F. graminearum* strain PH-1 and wheat variety "Norm" are used (Figure 2) (10). However, 48 and 72 h may be necessary for slower-growing mutants. Also, bulbous infection hyphae are often more prevalent after 48 h, as they stain very lightly after only 24 h.
6. In order to save supplies, we often include two glumes of the same treatment type per well when clearing/staining hyphae.
7. While we describe staining with lactophenol blue, we also have stained with calcofluor white and observed infection-related hyphae with fluorescent microscopy (10). Also, we have used a *F. graminearum* strain that constitutively expresses GFP (kindly provided by Dr. Jin-Rong Xu, Purdue University) and electron microscopy to observe these hyphal structures (unpublished data; 10).
8. Certain sections of the wheat glume appear to be more conducive to microscopic observation. Some parts (especially near the base of the glume or near the midrib) are too thick for mounting on a slide and/or background stain heavily with lactophenol blue.

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