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Perithecium Formation and Ascospore Discharge in *Fusarium graminearum*

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[Abstract] The filamentous ascomycete *Fusarium graminearum* (previously also known as *Gibberella zeae*) is a phytopathogen of grain cereals, reducing crop yield and grain quality. The abilities of sexual reproduction organ-perithecium formation, ascospore formation and discharge are all essential characteristics relevant to *F. graminearum* disease cycle. Here, we present the details of the protocol to study perithecium formation and ascospore discharge in *F. graminearum*.

Keywords: *Fusarium graminearum*, Sexual reproduction, Perithecium formation, Ascospore discharge

[Background] The ascomycete fungus *Fusarium graminearum* is the major causal agent of wheat *Fusarium* head blight and maize *Gibberella* stalk rot. This fungus can produce sexual fruiting bodies–perithecia on the surface of colonized host plants, the perithecia overwinters on crop debris and discharge ascospore for next year epidemic (Goswami and Kistler, 2004). Favored by moist and warm conditions, ascospores are forcibly discharged from perithecia and become airborne in air currents as the primary inoculum.

This fungus is homothallic; most strains can produce perithecia on carrot agar easily *in vitro* (Trail and Common, 2000). The microscopic study and a thorough description of perithecia development have been reported with temporal transcriptomic analysis during sexual development of *F. graminearum* (Trail and Common, 2000; Hallen *et al*., 2007). After induction of haploid hyphae at 0 h *in vitro*, dikaryotic cells formed and perithecium initiated at 24 h, young perithecia with central ascogenous cells and developing walls appeared at 48 h. The central ascospore matured at 144 h (Hallen *et al*., 2007). Studies have been conducted on factors that affect ascospore discharge and have concluded that relative humidity and temperature significantly affect the discharge process, while the light is not essential but it can help (Trail *et al*., 2002).

In this protocol, we outline the method of studying perithecium formation and ascospore discharge in *F. graminearum*, facilitating the identification of genes that have specific roles in sexual development and disease cycle.

Materials and Reagents

1. 60 mm x 15 mm diameter dish (Sigma-Aldrich, catalog number: P5481-500EA)
   Manufacturer: Excel Scientific, catalog number: D-901.
2. 2 ml microcentrifuge tube (BRAND, catalog number: 780546)
3. Glass spreading rod (CHEMGLASS, catalog number: CLS-1350-01)
4. Scalpel (Fisher Scientific, Fisherbrand™, catalog number: 08-920B)
5. Cork borer (Adelab Scientific, catalog number: LV-CRKBOR8)
6. Microscope slides (China Sail Brand, catalog number: 7105)
7. Sterile gauzes (Shanghai HongLong Medical Material Company)
8. Pipette tip box
9. Parafilm (Bemis, catalog number: 52858-000)
10. Fungal strain: *F. graminearum* PH-1 (NRRL 31084)
11. Sterile distilled H_2O
12. Ampicillin sodium salt (YEASEN, catalog number: 60203ES60)
13. TWEEN 60 (Sigma-Aldrich, catalog number: P1629)
14. Fresh carrot agar (see Recipes, store at 4 °C)
15. 2.5% TWEEN 60 (see Recipes, store at RT)

Equipment

1. 1 L Erlenmeyer Flask (Fisher Scientific, catalog number: S63274)
   Manufacturer: Corning, Pyrex®, model: 49801L/EMD.
2. 500 ml Erlenmeyer Flask (Fisher Scientific, catalog number: S63273)
   Manufacturer: Corning, Pyrex®, model: 4980500/EMD.
3. Autoclave (Zealway Instrument, model: GI54DWS)
4. Homogenizer (Ronghua Instrument Manufacturing, model: JJ-2B)
5. Mold incubator (Yiheng Instrument, model: MJ-150-I)
6. UV light (PHILIPS lighting, model: TL-D 15W BLB 1SL/25, catalog number: 928024810803)
7. Biological safety cabinet (Esco Micro, model: FHC1200A)
8. Induction chamber (JIANGNAN INSTRUMENT, model: GXZ-300)
9. Camera (Canon, model: EOS 7D)
10. Microscope (Olympus, model: BX51)

Procedure

A. Perithecia production on carrot agar
   1. Pick some mycelia from stock culture and inoculate carrot agar in the center of a 60 mm diameter plate (Figure 1A).

2. Place the inoculated plate in a mold incubator upright at 25 °C in the dark for 3-4 day until the mycelium reaches the edge of the plate (Figure 1B).

   Note: Some mutants of F. graminearum grow slower than wild-type and need more time to reach the edge of the plate.
3. Distribute 600 µl 2.5% TWEEN 60 to the surface of the plate (Figure 1C), press the aerial mycelium down with a sterile glass rod to make the surface of plate shiny without aerial mycelium (Figure 1D).
4. Place the plate under UV light (12 h on/12 h off) at 24 °C to induce perithecia formation (Figure 1E). Check the plate every day after induction, if the aerial mycelium appears again (Figure 1F), press down with 2.5% TWEEN 60 (50-200 µl) and sterile glass rod.
5. The surface of the plate should change from orange to red within 1-2 day after induction, (Figures 1G and 1H) and then to dark red 3-4 day after induction. The initial perithecia should be visible four days after induction.
6. The perithecia mature with ascospores usually on the 7th day after induction (Figures 1I and 3A).

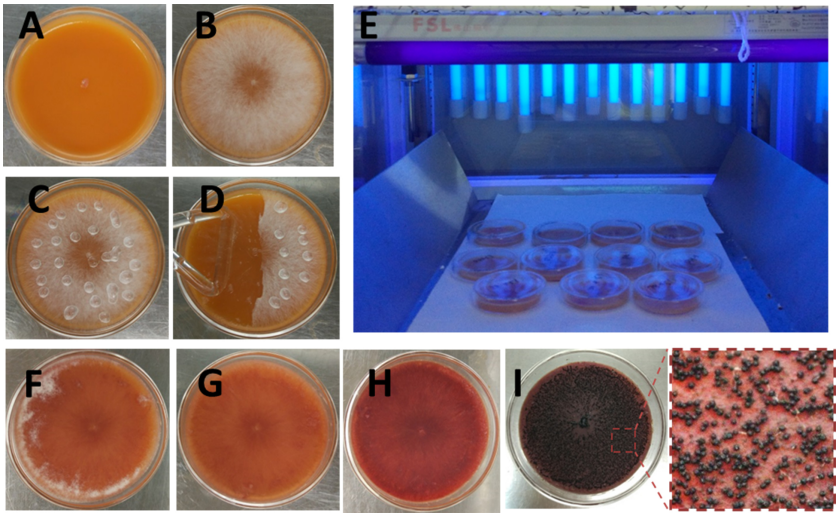

Figure 1. Perithecia formation on carrot agar. A. Inoculate carrot agar with mycelia. B. The colony reached the edge after three days post inoculation. C. Add TWEEN 60 on the surface of carrot agar. D. Press aerial mycelium down with a glass rod. E. The plate was placed under UV light in a chamber. F and G. Aerial mycelium appeared (F) and was pressed down (G) on one day after induction. H. The surface of plate changed from orange to red on two days after induction. I. The perithecia matured with ascospores on the 7th after induction.

B. Ascospore discharge analysis

1. On the 7th-day post induction, remove 10 mm diameter circles from the plate with mature perithecia using a cork borer (Figure 2A).
2. Using a scalpel, cut the circle into halves, place one of these halves on the edge of a glass microscope slide. Orient the halves on the slide to make sure the ascospore will be injected on

the slide (Figure 2B).

3. Add 50 µl sterile H_2O on the half piece (Figure 2B).
4. Fold sterile gauze to fill the pipette tip box and soak the box and gauze with water. Put the glass microscope slide with perithecia on the gauze in the box (Figure 2C). Close the box and seal the box with parafilm.
5. Put the box under UV light at 24 °C (Figure 2D); the ascospore is injected from the perithecia and accumulated at the front of perithecia after 12 h-24 h under the light (Figures 2E and 3C).
6. After the injected ascospore is photographed (Figure 2E), the ascospore can be washed off the slide with water for microscopic observation (Figure 2F).

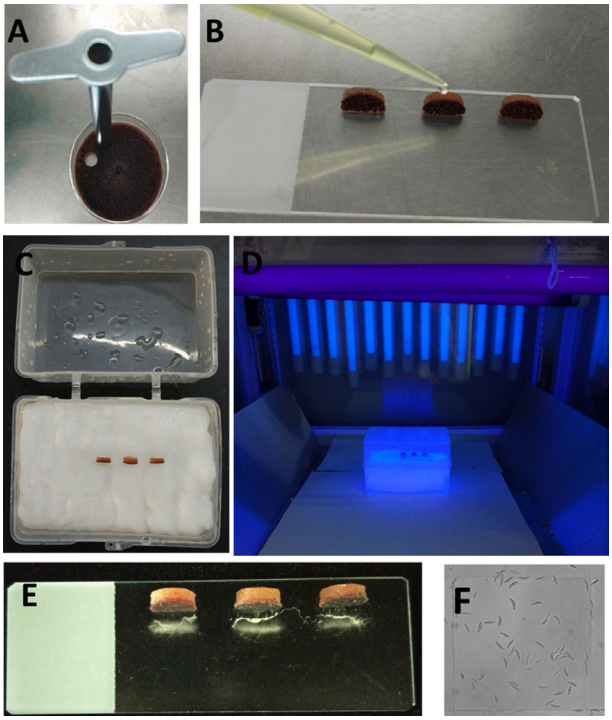

Figure 2. Ascospore discharge. A. Cut 10 mm diameter circle from the plate using a cork borer. B. Halves of the circles were placed on the glass microscope slide. Drop 50 µl water on the half piece. C. Place the glass microscope slide with perithecia on the gauze immersed in water. D. Put the box under UV light. E. The ascospores were discharged and accumulated on the glass slide. F. Microscopic observation of ascospores. Scale bar = 50 µm.

Note: There is a video presenting the methods of generating perithecia for further studies (https://www.jove.com/video/3895/sexual-development-and-ascospore-discharge-in-fusarium-graminearum). Our protocol was adapted from the video with modifications in fungal culture conditions, aerial mycelium press, perithecia induction conditions and ascospores discharge analysis. The video can be a visual reference to this protocol.

Notes

1. The critical steps in perithecia production are Steps A3 and A4 in Procedure section; please make sure the surface of the plate is shiny without aerial mycelium after pressing in Step A3 and check the plate every day after induction to avoid aerial mycelium growth in Step A4.
2. Please use fresh carrot agar; the carrot agar can't produce perithecia if it has been stored too long.

Data analysis

F. graminearum sexual development capability is often checked with perithecium production assay and ascospores discharge assay. Perithecium density and morphology are parameters for perithecium production assay (Figures 3A and 3B). Ascospores discharge assay includes observations of ascospores discharge or not and the morphology of ascospores (Figures 3C and 3D).

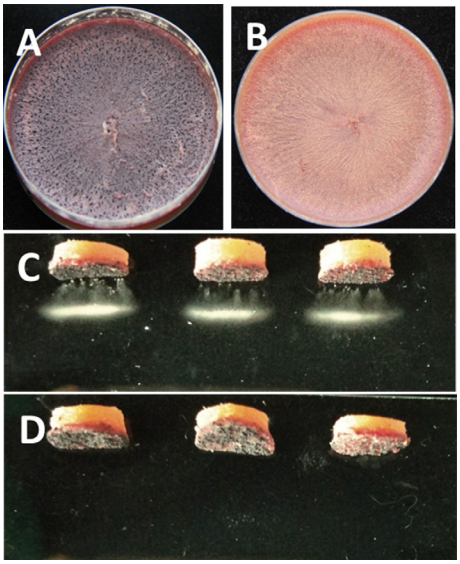

Figure 3. The perithecium formation and ascospore discharge analysis of wild-type and mutants with defects in perithecium development or ascospore discharge. A. Perithecium formation in wildtype PH-1. B. The mutant has defects in perithecium development cannot form perithecium on the surface of carrot agar. C. Ascospore discharge was normal in wild-type. D. The mutants had defects in ascospores discharge.

Recipes

1. Carrot Agar (1 L)
   a. Wash 400 g of fresh carrots, peel and cut into cubes
   b. Put the carrot cubes in one flask with 400 ml of water and autoclave at 121 °C for 15 min

c. Homogenize the carrot in a homogenizer until the mixture appears smooth
d. Add 500 ml of H_2O, mix thoroughly and divide into three flasks, supplement 2% agar and then autoclave at 121 °C for 15 min
e. Pour the carrot agar into 60 mm diameter plates with 100 mg/L ampicillin

Notes: The carrot agar can be stored at 4 °C for up to one month.

2. 2.5% TWEEN 60 (100 ml)
   a. Weighing 2.5 g semi-solid TWEEN 60 in a 2 ml centrifuge tube
   b. Mix with100 ml H_2O and autoclave at 121 °C for 15 min
   c. Store at room temperature, and it is stable for years

Acknowledgments

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References

1. Cavinder, B., Sikhakolli, U., Fellows, K. M. and Trail, F. (2012). Sexual development and ascospore discharge in *Fusarium graminearum*. *J Vis Exp* (61): 3895.
2. Goswami, R. S. and Kistler, H. C. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol Plant Pathol* 5(6): 515-525.
3. Hallen, H. E., Huebner, M., Shiu, S. H., Guldener, U. and Trail, F. (2007). Gene expression shifts during perithecium development in *Gibberella zeae* (anamorph *Fusarium graminearum*), with particular emphasis on ion transport proteins. *Fungal Genet Biol* 44(11): 1146-1156.
4. Trail, F. and Common, R. (2000). Perithecial development by *Gibberella zeae*: a light microscopy study. *Mycologia* 92(1): 130-138.
5. Trail, F., Xu, H., Loranger, R. and Gadoury, D. (2002). Physiological and environmental aspects of ascospore discharge in *Gibberella zeae* (anamorph *Fusarium graminearum*). *Mycologia* 94(2): 181-189.

