

Video Article

Sexual Development and Ascospore Discharge in *Fusarium graminearum*

Brad Cavinder¹, Usha Sikhakolli², Kayla M. Fellows³, Frances Trail^{2,4}

¹Genetics Program, Michigan State University

²Department of Plant Biology, Michigan State University

³Human Biology Program, Michigan State University

⁴Department of Plant Pathology, Michigan State University

Correspondence to: Frances Trail at trail@msu.edu

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Abstract

Fusarium graminearum has become a model system for studies in development and pathogenicity of filamentous fungi. *F. graminearum* most easily produces fruiting bodies, called perithecia, on carrot agar. Perithecia contain numerous tissue types, produced at specific stages of perithecium development. These include (in order of appearance) formation of the perithecium initials (which give rise to the ascogenous hyphae), the outer wall, paraphyses (sterile mycelia which occupy the center of the perithecium until the asci develop), the asci, and the ascospores within the asci¹⁴. The development of each of these tissues is separated by approximately 24 hours and has been the basis of transcriptomic studies during sexual development^{12,8}. Refer to Hallen *et al.* (2007) for a more thorough description of development, including photographs of each stage. Here, we present the methods for generating and harvesting synchronously developing lawns of perithecia for temporal studies of gene regulation, development, and physiological processes. Although these methods are written specifically to be used with *F. graminearum*, the techniques can be used for a variety of other fungi, provided that fruiting can be induced in culture and there is some synchrony to development. We have recently adapted this protocol to study the sexual development of *F. verticillioides*. Although individual perithecia must be hand picked in this species, because a lawn of developing perithecia could not be induced, the process worked well for studying development (Sikhakolli and Trail, unpublished).

The most important function of fungal fruiting bodies is the dispersal of spores. In many of the species of Ascomycota (ascus producing fungi), spores are shot from the ascus, due to the generation of turgor pressure within the ascus, driving ejection of spores (and epiplasmic fluid) through the pore in the ascus tip^{2,7}. Our studies of forcible ascospore discharge have resulted in development of a "spore discharge assay", which we use to screen for mutations in the process. Here we present the details of this assay.

F. graminearum is homothallic, and thus can form fruiting bodies in the absence of a compatible partner. The advantage of homothallism is that crossing is not necessary to generate offspring homozygous for a particular trait, a facet that has facilitated the study of sexual development in this species^{14,7}. However, heterothallic strains have been generated that can be used for crossing^{5,9}. It is also possible to cross homothallic strains to obtain mutants for several genes in one strain¹. This is done by coinoculating one Petri dish with 2 strains. Along the meeting point, the majority of perithecia will be recombinant (provided a mutation in one of the parent strains does not inhibit outcrossing). As perithecia age, they exude ascospores en masse instead of forcibly discharging them. The resulting spore exudate (called a cirrhous) sits at the tip of the perithecium and can easily be removed for recovery of individual spores. Here we present a protocol to facilitate the identification of recombinant perithecia and the recovery of recombinant progeny.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3895/>

Protocol

1. Producing Perithecia

1. Center inoculate carrot agar¹⁰ in a 6.0 cm diam Petri dish with a fertile strain of *F. graminearum* (Recommend PH-1).
2. Place inoculated dishes under bright fluorescent lights at room temperature (18-24 °C) and allow to grow until mycelium has reached the outer edge of the Petri dish (3-5 days). Commercial household fluorescent lights work fine for stimulating fruiting body formation and discharge.
3. Gently remove the aerial mycelia with a sterile toothpick.
4. Distribute 1.0 ml 2.5 % Tween 60 aq to the surface with a sterile glass hockeystick or rounded end of a sterile glass rod.
5. Return plates to light. Do not add Parafilm to plates.
6. After 24 hr, the surface of the plates should have a shiny appearance. If mycelia reappears, scrape surface and re-apply Tween-60 solution.

7. Observe development of perithecia over the next week. The intermediate stages of perithecium development can be harvested by gently scraping the surface of the agar and flash frozen for RNA extraction (**Figure 1**).
8. Mature spores will accumulate on the lid of the plate by day 7. Initially these will only be visible under a dissecting microscope, but by day 9, they will have accumulated to sufficient density so as to be visible to the naked eye.

2. Ascospore Discharge Assay

1. On day 6 after application of Tween solution, cut a 1 cm diam circle out of the agar with a wood borer. Alternatively, a scalpel can be used to remove a similar sized segment. The circle can be sliced in half and each half placed on a glass microscope slide.
2. Orient the pieces so the surface containing the fruiting bodies is perpendicular to the surface of the slide, and the spores are fired down the length of the slide.
3. Place the slide in a humidity chamber overnight under lights. Spores will accumulate on the slide and be visible to the naked eye the next morning (**Figure 2**).
4. Accumulated spores may be washed off the slide with water and quantified if desired.
5. Potential ascospore discharge inhibitors can be assayed by adding them to the back side of the agar block during assembly of the assay. Some ion channel inhibitors that reduce discharge have been previously assayed with this technique¹⁵.

3. Generation of Recombinant Progeny

1. Initiate cross by inoculating carrot agar with two strains of *F. graminearum* (one Nit⁺ and one nit⁻), placing the inoculation points on opposite sides of the Petri dish.
2. Once hyphae have filled the surface, scrape off aerial portion and apply Tween-60 solution as described above. Use a marker to note on the side of the Petri dish where the hyphae meet.
3. Return cultures to light and incubate until perithecia have developed, and cirrhi have begun to form from the perithecia along the intersection between the two cultures.
4. Under the dissecting microscope, bring into view the perithecia along the intersection of the two cultures. Using an insect pin, sterilized and dipped into sterile water, touch a cirrhus along this border lightly with the tip of the pin. The moisture on the pin should allow the cirrhus to stick to the pin.
5. Rinse the tip of the pin with the cirrhus on it in 200 μ l sterile water in an Eppendorf tube to wash the spores off.
6. Flame the pin, moisten it again and pick another cirrhus. Pick 10 to 15 cirrhi, and place each in a separate tube of water.
7. Briefly vortex tubes containing suspended cirrhi.
8. Remove 60 μ l of the spore suspension with a pipette and place on MMTS medium¹ in a 9 cm Petri dish. Spread with a sterile hockey stick. The remaining spore suspension may be stored at 4 °C for up to a week and replated if necessary.
9. Incubate at room temperature (light not necessary) for 3-5 days until very small colonies appear (**Figure 3**). There will be two kinds of phenotypes among the colonies. The nit⁻ colonies will have sparser hyphae than the Nit⁺ colonies. Cirrhi from recombinant perithecia will show both colony phenotypes in approximately equal proportion. Discard the plates with colonies of only a single phenotype on them. Note that sometimes more than two phenotypes result due to segregation of other factors.
10. Move colonies to V8 or carrot agar for storage. Test colonies on Czapek's Dox Agar (the nit⁻ strains will not grow on Czapek's Dox) and on PDA with Chlorate (0.5 M Potassium Chlorate; the Nit⁺ strains will not grow on PDA with Chlorate) to confirm the correct phenotype. Examine these colonies for the desired qualities.

4. Representative Results

Producing perithecia

The goal is to have a lawn of perithecia without any mycelia or spores apparent. The surface of the culture will appear similar to black velvet (**Figure 1**). At 24 hr after the application of Tween the surface of the agar should have a slight sheen. If mycelia reappear, then the plate may not have been scraped enough to induce perithecium development.

Ascospore discharge

Always provide in your assay several blocks of agar from the wild-type strain. If those do not fire, then either your perithecia are too young or too old. If they are too old, then numerous hyphae will appear over the surface of the culture giving it a whitish hue. If this is not the case, they may be too young, and the assay should be tried again in 24 hr. Occasionally, cultures do not discharge well, and the experiment will have to be repeated. Be sure you perform the assay under the proper light conditions.

Recombinant progeny

Recombinant perithecia should make up the majority of perithecia along the intersection between the two strains. We usually process 10 cirrhi from a cross and at least 4-6 of those should be recombinant. Very occasionally, a mutation prevents a strain from mating. If one of the parents has an aberrant growth phenotype, then more than 2 phenotypes may be present among the colonies on MMTS.

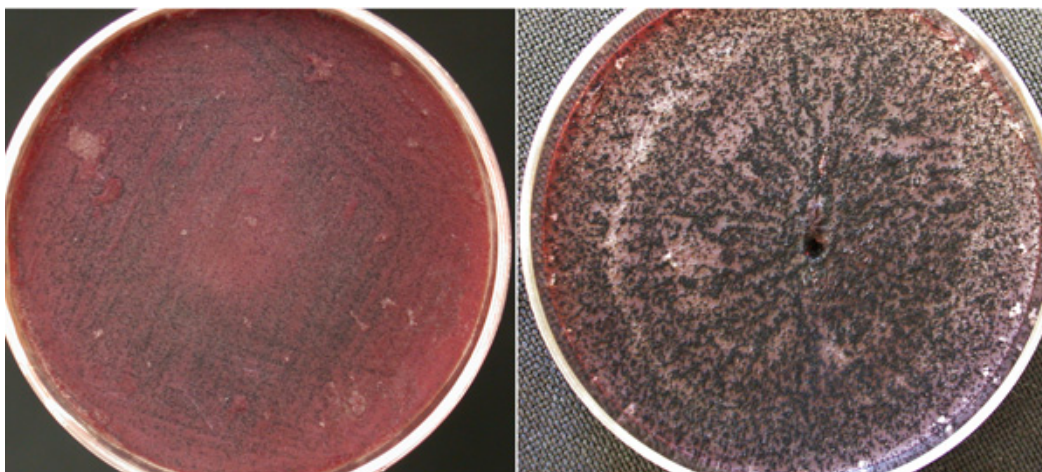


Figure 1. Stages of perithecium development on carrot agar. Left, 72 h after application of Tween. Note red pigment and very young perithecia visible as a black grains on the surface. Right, at 144 h, mature perithecia have formed. Note near-complete absence of superficial mycelia in both cultures.



Figure 2. Discharge assay. The orange carrot agar plug is oriented so that spores forcibly discharged from the mature perithecia on its surface can accumulate on the surface of the glass slide. 15 hr accumulation time.

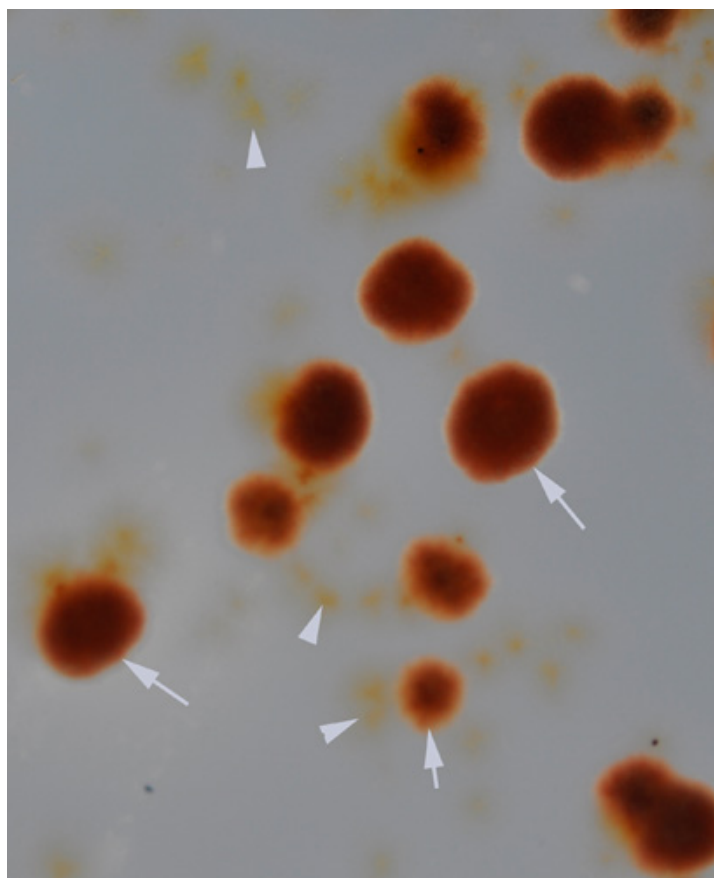


Figure 3. Differences in growth between Nit⁺ and nit⁻ colonies on selective MMTS medium. The nit⁻ colonies (arrowheads) are smaller. The Nit⁺ colonies (arrows) show robust growth. Photograph taken after 7 days.

Discussion

F. graminearum is particularly well adapted to the study of fruiting body development due to the availability of a well-annotated genome (mips.helmholtz-muenchen.de/genre/proj/FGDB/; ⁴), and the availability of an Affymetrix-based microarray⁶. This has facilitated the ability to identify genes important for ascospore discharge^{7,11,3}. The methods presented here will allow the researcher to focus on a discrete set of developmental stages and functions for genetic analysis of the fruiting bodies of *F. graminearum*. The methods are also easily adaptable to related fungi that can be induced to fruit in culture, and can be used as a standard for development in a variety of other fungal fruiting body types.

The ability to assess a spore firing phenotype can be subtle in species where spores are small and hard to see, such as *F. graminearum*. The collection of spores on a clean slide facilitates both visual assessment, and quantitative assessment, as spores may be washed off the slide and quantitated. Some fungal species produce a mucilage that surrounds their spore and the spores cannot be rinsed off, but must be counted microscopically as they remain on the slide. We have found this to be true of *Sclerotinia sclerotiorum*.

Disclosures

We have nothing to disclose.

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