Video Article

Investigating Teliospore Germination Using Microrespiration Analysis and Microdissection

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Abstract

Smut fungi are the etiological agents of several devastating agricultural diseases. They are characterized by the production of teliospores, which are thick-walled dispersal agents. Teliospores can remain dormant for decades. The dormancy is characterized by low metabolic rates, paused macromolecular biosynthesis and greatly reduced levels of respiration. Upon receiving required environmental signals, teliospores germinate to produce haploid cells, which can initiate new rounds of infection. Teliospore germination is characterized by the resumption of macromolecular biosynthesis, increased respiration and dramatic morphological changes. In order to precisely measure changes in cellular respiration during the early stages of germination, we have developed a simple protocol employing a Clark-type respirometer. The later stages of germination are distinguished by specific morphological changes, but germination is asynchronous. We developed a microdissection technique that enables us to collect teliospores at distinct germination stages.

Video Link

The video component of this article can be found at https://www.jove.com/video/57628/

Introduction

The smut fungi (*Ustilaginales*) consist of over 1,600 species that infect grasses including the important cereal crops of corn, barley, and wheat, causing billions of dollars in crop losses annually[1]. These fungi are characterized by the production of teliospores, which have darkly pigmented cell walls and are the dispersal agents. Teliospores function to shield genetic material during the stresses of dispersal between host plants, and can persist in a dormant state for years[2]. As such, teliospores are an essential component of disease spread.

In order to study teliospore biology, our laboratory utilizes the model smut fungus *Ustilago maydis* (*U. maydis*), which is the causal agent of the disease 'common smut of corn'. Mature *U. maydis* teliospores are characterized by growth arrest, reduced cellular metabolism, and low levels of cellular respiration[3]. In favorable environmental conditions (*e.g.*, the presence of specific sugars), *U. maydis* teliospores germinate and complete meiosis, producing basidiospores which can initiate new rounds of infection. Germination is characterized by increased respiration, the return to metabolic activity, and the progression through observable morphological stages of germination[4].

The initial stage of germination includes increased respiration and metabolic function, however, there are no morphological indications of change. The original measurements of respiratory change in *U. maydis* were carried out over 50 years ago, measuring oxygen consumption manometrically with a Warburg flask apparatus[5]. We have developed a new, simple method of studying precise changes in respiration during teliospore germination by measuring oxygen consumption over a time course of germination using a Clark-type microrespirometer. We previously used this method to study changes in respiratory rate between wild-type *U. maydis* haploid cells and mutants with defective mitochondria[6], and have adapted the protocol here to study changes in teliospore respiration during germination. This provides a means of accurately identifying the timing of respiration change so that we can target teliospores at the appropriate time after the initiation of germination to investigate early molecular events. The progression of germination can be followed microscopically once the promycelia emerges from the teliospore, but the asynchronous nature inhibited the isolation of enough teliospores at a given stage for investigation. We developed a microdissection technique similar to those used for *in vitro* fertilization to physically collect teliospores at distinct morphological stages of germination.

Protocol

1. Corn Cob Infection

1. Grow *Zea mays* (cv. Golden Bantam) until cobs are formed and have started to silk (approximately 60 days).

2. Culture compatible haploid *U. maydis* strains using standard protocols as previously described[7].
3. Infect corn cobs using standard protocols as previously described[7].

2. Teliospore Harvesting

1. Autoclave equipment (Büchner funnels, Büchner flasks, blenders, 250 mL centrifuge bottles, flat spatulas, and water) using a standard dry cycle with at least 30 min sterilization at 121 °C (standard liquid cycle for water).
2. Remove infected cobs from plants (approximately 28–35 days post infection) using a razor and set the cobs on a tray covered in bench protector.
3. Remove the tumours from cobs with a razor blade and collect in a beaker.
4. Fill a 250 mL laboratory blender cup with tumours until approximately 1/3 full and add autoclaved dH_2O until the blender cup is approximately 3/4 full. Disrupt the tumours by pulsing the blender at low, until homogenized.
5. Connect the vacuum pump to a water trap and then to a 1 L Büchner flask.
6. Insert large Büchner funnel into the flask and line the bottom of the Büchner Funnel with four layers of cheesecloth.
7. Turn on the vacuum pump.
8. Pour a portion of the homogenized tumours through the cheesecloth and scrape with a spatula.
9. Pour some dH_2O into the cheesecloth in order to flush the teliospores through.
10. Repeat until the dH_2O coming through the cheesecloth is clear.
11. Wring out the cheesecloth containing the homogenized tumour material into the filter to ensure maximum teliospore recovery.
12. When the 1 L Büchner flask is getting close to full, empty it into a large Erlenmeyer flask and set it aside.
13. Put a new piece of cheesecloth into the filter and repeat the steps (2.7–2.12) until all of the tumours have been disrupted and filtered.
14. Pour the filtered teliospores into autoclaved 250 mL centrifuge bottles and centrifuge at 1,000 x g for 5 min, and decant the supernatant.
15. Repeat step 2.14 until all of the filtered teliospores are pelleted by centrifugation.
16. Suspend the pellets in a small amount of water and transfer to 50 mL centrifuge tubes.
17. Centrifuge the tubes at 1,000 x g for 5 min, and decant the supernatant.
18. Suspend the pellet in approximately 50 mL of dH_2O, centrifuge the tubes at 1,000 x g for 5 min, and decant the supernatant. Gently scrape off the gray top layer with a spatula and dispose of it. Repeat until there is no longer a gray layer on top.
19. Dry the samples overnight in a vacuum desiccator.
20. Store dried teliospores at 4 °C until use.
21. **If desired, treat the teliospores with copper sulphate[8] before inducing to germinate. If not treated, then perform thorough microscopic analysis of the teliospores to confirm the sample represents pure teliospores and is devoid of bacterial or other contaminations.**
    1. Weigh out approximately 50 mg of teliospores in a 1.5 mL microcentrifuge tube.
    2. Add approximately 1.0 mL of 0.75% $CuSO_4$ to the 1.5 mL microcentrifuge tube containing the teliospores. Pipette up and down to suspend the teliospores in the $CuSO_4$ solution followed by agitating the sample for 3 h.
    3. Centrifuge the sample at 2,500 x g for 5 min and remove the supernatant. Resuspend the teliospore pellet with sterile water, repeat the centrifugation, and remove the supernatant.
    4. Repeat step 2.21.3 two more times.

3. Teliospore Viability and Germination Test

1. Weigh out approximately 10 mg of *U. maydis* teliospores in a 1.5 mL microcentrifuge tube to assess their viability and the timing of germination.
2. In a biosafety cabinet, prepare potato dextrose broth (PDB, 24 g/L) supplemented with streptomycin sulfate (160 µg/mL).
3. Suspend the teliospores in 500 µL of PDB. Gently pipette to mix and break up all clumps of teliospores.
4. Transfer the teliospore suspension to an autoclaved 250 mL Erlenmeyer flask containing 10 mL of the PDB.
5. Incubate the flask at 28 °C shaking at 90 rpm for 12–16 h.
6. In a biosafety cabinet, remove a 20 µL sample of the teliospores induced to germinate and prepare a microscope slide.
7. **Using a microscope, visually assess stages of germination that are present and the presence of bacterial contamination.**
    1. Count the number of teliospores at stages I through V using a hemocytometer and determine the percent that have germinated.
    2. If only stage I teliospores are present, continue to incubate the flask for a total of 24 h before assessing teliospore germination. Continue incubation for a maximum of 48 h before deeming the sample non-viable.
    3. If bacterial contamination is present, supplement the PDB with kanamycin sulfate (50 µg/mL) as well as streptomycin sulfate (160 µg/mL) and then repeat steps 3.1 to 3.7. If bacterial contamination persists, treat teliospores with copper sulfate and repeat steps 3.1 to 3.7.

4. Induction of Germination for Respiration Monitoring

1. Weigh equal amount (*e.g.*, 50 mg) of teliospores for each respiration experiment.
2. In a biosafety cabinet, add teliospores to an autoclaved respiration chamber.
3. Fill the chamber with PDB (24 g/L) supplemented with streptomycin sulfate (160 µg/mL) and kanamycin sulfate (50 µg/mL).
4. Pipette up and down to create a teliospore suspension.
5. Place the chamber lid in the chamber to create air tight seal.

5. Obtaining Oxygen Consumption Rate (OCR) Measurements

1. Place the chamber in the chamber rack inside a water bath (preheated to 28 °C).
2. Place the O_2 probe inside the opening of the chamber.
3. Monitor the data points appearing in real time on the "SensorTrace Rate" program, and let the probe stabilize (~3 min after the probe is placed in the chamber).
4. Click "Measure" to measure O_2 levels continuously for 6 h with measurements recorded at 2 s intervals.
5. Stop the measurement, and repeat steps 4.1–5.4 for each sample to be analyzed.
6. Export the data to Microsoft Excel by clicking "File | Export | Save as .xls".

6. Data Analysis

1. **Calculate OCR**
    1. In the exported Excel file under the "Within_Rates" tab, record the "Rate" for each chamber measurement (nmol/h).
    2. For each experimental sample, subtract the "Rate" of the blank chamber from the "Rate" of the experimental sample chamber to obtain a corrected OCR value, and take the absolute value of this number.
    3. Calculate the total OCR per mg of teliospores by dividing the corrected absolute OCR value by the cellular mass used.
    4. Average replicate "OCR per mg of teliospores" values for each strain.
2. Analyze the data using appropriate statistical method (*e.g.*, student's *t*-test, analysis of variance) using Microsoft Excel of other statistical software.
3. To graph raw data, calculate the percentage of oxygen remaining for each time-point you wish to graph. Divide first reading by itself and multiply by 100 (100% oxygen remaining), then divide each subsequent reading by the first reading, and multiply by 100 to obtain the percent of oxygen remaining in the chamber.

7. Induction of Teliospore Germination to Isolate Teliospores at Distinct Stages of Germination

1. Prepare PDB (24 g/L) supplemented with streptomycin sulfate (160 µg/mL) in a biosafety cabinet.
2. Place approximately 10 mg of *U. maydis* teliospores into a 1.5 mL microcentrifuge tube.
3. Suspend the teliospores in 500 µL of PDB. Gently pipette to mix until there are no clumps of teliospores in the medium.
4. Transfer the teliospore suspension to an autoclaved 250 mL Erlenmeyer flask containing PDB supplemented with streptomycin sulfate.
5. Incubate the flask overnight at 28 °C shaking at 90 rpm.

8. Preparation of Petri Dish and Micromanipulator

1. **Prepare a Petri dish (57 cm^2) by pipetting rows of droplets for microcapillary preparation, and sample collection.**
    1. Pipette 5 µL (x4) dH_2O droplets across the top of the Petri dish.
    2. Pipette 2 µL (x3) of RNA stabilization solution on the Petri dish to be used for sample collection.
    3. Pipette 5 µL (x30) droplets of germinating teliospores on the Petri dish.
2. Add 15 mL of mineral oil to the petri dish. Ensure that all droplets are covered by oil before proceeding.
3. Prepare a microcapillary with a 15 µm inner diameter, 1 mm flange, 55 mm length, and a 20° tip angle by placing it in the microcapillary holder and submerging it in the mineral oil where capillary action will allow the mineral oil to enter the microcapillary. Release the pressure in the microcapillary before bringing it to the water droplet. Aspirate to prepare the microcapillary with water.

9. Isolation of Stage-specific Germinating Teliospores

1. Using the controls of the micromanipulator, move the prepared microcapillary to one of the germination droplets. Penetrate the droplet, lower the microcapillary, and bring the mouth of the microcapillary up to a germinating teliospore at the stage of germination of interest.
2. Slowly aspirate to capture the germinating teliospore. Stop aspirating once the teliospore has entered the microcapillary. Repeat until there are approximately five teliospores in the microcapillary.
3. Raise the microcapillary with the micromanipulator and bring it to the collection droplet of RNA stabilization solution. Penetrate the droplet and inject the teliospores into the droplet.
4. Repeat steps 8.1 to 8.3 until approximately 1,000 teliospores have been captured.

10. Recovery of Collection Droplet

1. Pipette up the collection droplet and transfer it to the lid of an RNase/DNase-free 2.0 mL microcentrifuge tube. Carefully remove the mineral oil with a pipette without disturbing the collection droplet.
2. Use the teliospores for downstream applications such as RNA isolation.

Representative Results

Using the Clark-type microrespirometer-based method of measuring changes in respiration during teliospore dormancy and germination, we confirmed that dormant teliospores exhibit a low level of respiration (~1,075 µmol/h/mg) compared to germinating teliospores (~2,614 µmol/h/mg; **Figure 1A**). This represents a ~2.4-fold change in average rate of respiration between dormant teliospores and teliospores that have been induced to germinate. In addition, we have identified that teliospores that have been induced to germinate have a ~45 min delay in oxygen uptake (**Figure 1B**). This is indicated by the ~30 min delay in oxygen uptake (**Figure 1B**) in addition to the ~15 min delay between the induction of germination and start of oxygen measurements. This identifies a time point to begin assessing molecular changes in the germinating teliospores that are not visibly changing.

Subsequent changes during germination can be observed microscopically. Five stages of germination were determined. Stage I of germination represents teliospores that have been induced to germinate but remain indistinguishable from dormant teliospores. Stage II teliospores have an emerging promycelium with a length that is less than or equal to the diameter of the teliospore. Stage III teliospores have promycelia that are greater than the teliospore diameter. Stage IV of germination is the initial budding of basidiospores from the promycelia, and Stage V are the resulting haploid basidiospores that divide by budding (**Figure 2**). Using the microdissection technique that we have developed, we have successfully isolated 500 to 1,000 germinating teliospores for downstream applications such as RNA isolation for RT-PCR or RNA-Seq (**Table 1**). **Figure 3** shows the general set up of the Petri dish for microdissection and the steps for microdissection using a micromanipulator.

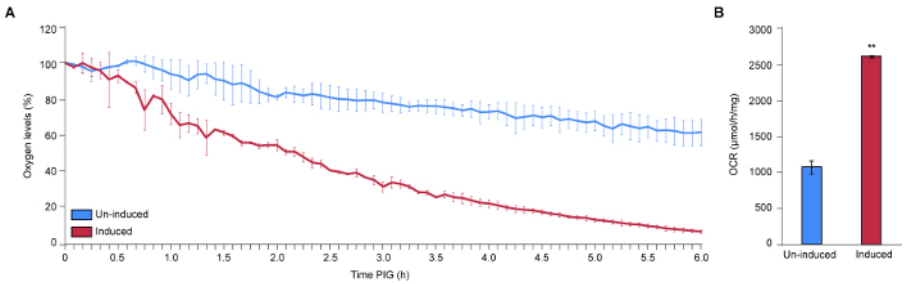

Figure 1: Time course of oxygen consumption during teliospore germination. Dormant teliospores were induced to germinate, and oxygen levels were recorded continually for 6 h using a Clark-type microrespirometer. Un-induced dormant teliospores were used as a control, and all measurements were normalized to a blank sample. **(A)** Data represented as average OCR. **(B)** Raw data plotted to obtain respiration curves, permitting the detection of changes in OCR during the time course. Teliospores that have been induced to germinate consume oxygen at an average rate 2.4-fold faster than un-induced dormant teliospores (p <0.01; Student's t-test). PIG: post-induction of germination. Please click here to view a larger version of this figure.

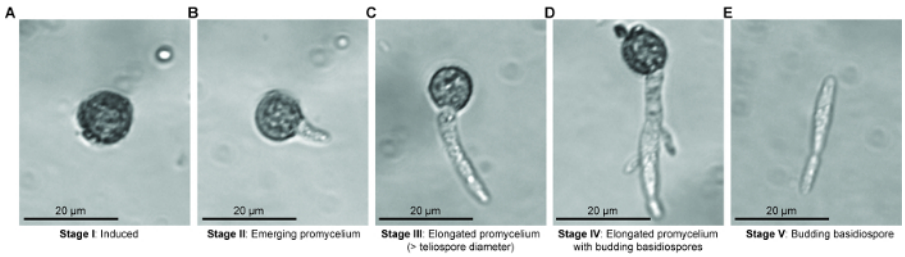

Figure 2: Stages of teliospore germination. Stages I through V of teliospore germination are illustrated **(A-E)**. Scale bar = 20 µm. Please click here to view a larger version of this figure.

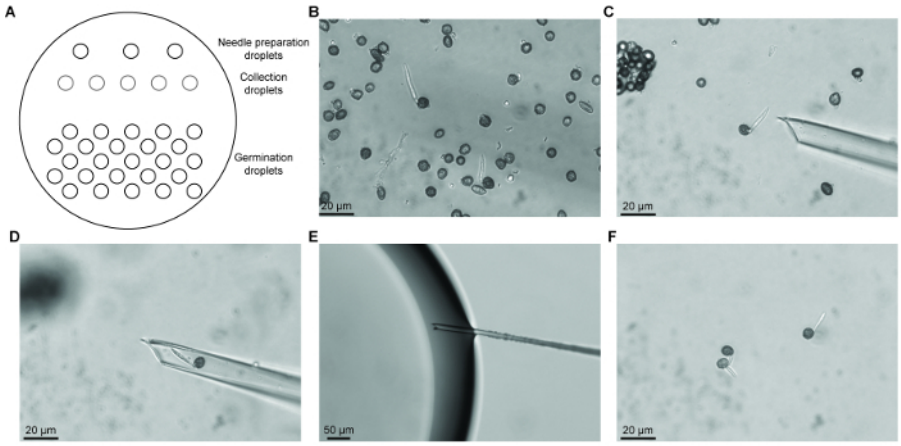

Figure 3: Microdissection to isolate distinct morphological stages of germinating teliospores. The general set up of a Petri dish for microdissection and the steps for isolating teliospore at stage III of germination are illustrated. **(A)** Illustration of a Petri dish set up with rows of droplets containing either sterile water, RNA stabilization solution, or germinating teliospores. Following germination induction, teliospores were isolated at specific stages of germination using microdissection. **(B)** A germination droplet containing induced to germinate teliospores in Stages I through III. **(C)** A prepared microcapillary was brought up to a Stage III teliospore for collection through aspiration. **(D)** The Stage III teliospore in a glass microcapillary is removed from the germination droplet and moved to a collection droplet. **(E)** The microcapillary was inserted into the collection droplet containing RNA stabilization solution and the Stage III teliospore was injected into the droplet. **(F)** A collection of Stage III teliospores in the RNA stabilization solution. Scale bar = 20 µm (A-D, F) and 50 µm (E). Please click here to view a larger version of this figure.

Germination Stage	Number of germinating teliospores isolated
Stage I	1,000
Stage II	500
Stage III	650

Table 1: Number of germinating teliospores successfully isolated for each germination stage in a standard isolation experiment. The table illustrates average numbers of teliospores that have been isolated using microdissection for Stages I through III before collection for downstream applications.

Discussion

Basidiomycete biotrophic plant pathogens cause billions of dollars in crop losses annually. The vast majority of these pathogens produce teliospores that are integral to fungal dispersal and sexual reproduction. Gaining knowledge of the development and germination of teliospores is critical to understanding the spread of the devastating diseases caused by these fungi. In order to identify molecular changes at key control points we have devised a method to identify the timing of physiological shifts and another to isolate teliospores at distinct stages of germination. Seto *et al.* (unpublished) noted five stages of teliospore germination by light microscopy (**Figure 2**). In order to investigate physiological activation during Stage I and to assess respiration rate during germination, we used a Clark-type microrespirometer to precisely measure changes in oxygen consumption. Our sample data indicate that our method is precise and highly reproducible. Our findings confirm that germinating *U. maydis* teliospores exhibit a drastic increase in cellular respiration compared to un-induced dormant teliospores. For the first time, we have identified that *U. maydis* teliospores that have been induced to germinate exhibit a ~45 min delay in oxygen uptake. This suggests that *U. maydis* teliospores may require some time to process germination signals (*e.g.*, the presence of sugars) before responding, that increased oxygen uptake is not among the very immediate responses to germination signals or that our assay was not sensitive enough to detect the minimal change in initial oxygen uptake.

Previous studies examining respiration rates of smut teliospores[3] relied on a Warburg flask apparatus to measure oxygen levels manometrically[5]. Briefly, this method measures oxygen consumption and CO_2 production by detecting changes in pressure in an enclosed flask through the direct observation of fluid level changes in the manometer arm. The experiments can be difficult to set up, and measurements can be imprecise. The apparatus must be attended throughout the period of measurement and, extensive calculations are required to estimate OCR. Our protocol makes use of technological advances, eliminating the requirement for the user to remain by the apparatus for the duration of the experiment, take measurements by eye, and use extensive mathematical formulas. Others have used early Clark-type respirometers to measure OCR of *Neurospora crassa*[9] and *Botryodpilodia theobromae*[10] spores, however, these early instruments permitted continuous measurements for a maximum of 20 min. This limitation would not have allowed the identification of the ~45 min delay in oxygen uptake we observed with the newer model respirometer. Our protocol has made data interpretation simpler, as the readout is the concentration of oxygen remaining in the chamber, which can be directly graphed without any calculations or data manipulation. In addition, it is possible to take continuous measurements (every 2 s) for an indefinite amount of time until available oxygen is completely depleted. This permits the identification of small changes in respiration over a long period of time. Therefore, we have improved upon earlier techniques and developed a simple, precise, and reproducible method to measure oxygen consumption of fungal spores. To our knowledge, this is the first study to use a modern Clark-type respirometer to study respiration of dormant versus germinating teliospores of smut fungi.

Despite the ease and simplicity of this protocol, optimization is required and there are biological realities that limited the analysis. First, appropriate sample sizes must be identified to achieve reasonable OCRs. Too much sample can lead to premature crashing of oxygen levels,

and too little sample can result in the inability to observe meaningful changes in oxygen consumption. Second, it is imperative to allow the probe time to stabilize (~3 min) to provide accurate initial data. Lastly, it is important to supplement germination medium with antibacterial agents (*e.g.*, streptomycin sulfate) in order to ensure bacterial contamination does not alter OCR readings. The biological limitations we faced were a low germination rate over the time course of measurement, (~1%) as determined by observing visual morphological changes. Determining spore viability would allow this rate determination to be converted to a rate per spore number and isolating teliospores with higher rates of germination would lead to higher OCRs. The asynchronous germination of *U. maydis* teliospores[11] is a reality that must be accounted for and may have contributed to an inability to detect oxygen consumption earlier in germination.

In order to improve the accuracy and precision of measuring changes in teliospore respiration, future adaptations to this method could include measuring OCR on a single cell-basis. Micromanipulation techniques could be used to isolate a single teliospore, which can then be induced to germinate, and its respiration rate can be monitored. This could improve resolution, providing information regarding the OCR during the dormancy-germination shift per teliospore, rather than per mg of teliospores. In addition, this would solve the confounding issue of asynchronous germination.

For later stages of germination, we developed a micromanipulation method to isolate teliospores at common stages of germination. This allowed the creation of relatively synchronous teliospore populations for analysis. Various methods for isolating single microorganisms have been described and have been improved upon over the years[12]. These methods include the dilution of spore suspensions to obtain single microorganisms, semi-mechanical methods with the use of microcapillaries to obtain spores that are transferred to medium for culturing, and mechanical methods which use micromanipulators. Previous methods that we used to obtain teliospores at the same stage of germination include counterflow centrifugal elutriation and filtering germinating teliospores through a nylon membrane with a specific pore size. Using these methods allowed us to enrich for germinating teliospores, however, our samples still contained teliospores in various stages of germination[13]. Current technology for micromanipulation of single microorganisms has improved with the introduction of higher magnification and instruments for fine control of capillary needles, aspiration, and transfer of microorganisms. Previous micromanipulation techniques have focused on isolating single cells for culturing or for use in single cell PCR applications[14]. The use of micromanipulators to isolate single fungal spores has not previously been established. A previous method for isolating single fungal spores involved the use of fine forceps or needles to pick small pieces of solid medium containing germinating spores[15]. Micromanipulation with the use of micromanipulators is widely used in yeast studies where clusters of ascospores can be separated following sporulation in culture on agar medium for meiotic genetic analysis[16]. We have developed a method which combines the micromanipulation technique for bacterial cells[14] and *in vitro* fertilization methods for isolating germinating teliospores. We have shown that hundreds of common germination stage teliospores can be obtained with this technique. These samples can be used for downstream expression studies using techniques such as RT-qPCR or RNA-seq. Obtaining a population of teliospores in which germination is synchronized permits the analysis of specific changes in gene expression that occurs during early, mid and later stages of teliospore germination.

Microdissection of stage specific germinating teliospores may require experience in set up and recognizing the different stages of germination, however, this experience can be obtained quickly through practice. There are several steps that must be followed for successful microdissection followed by RNA isolation. First, germination medium must be supplemented with antibiotics (*e.g.*, streptomycin sulfate) to suppress the growth of bacterial contamination when germination is initiated as well as during collection of germinating teliospores. Second, it is important to use a stabilization solution to stabilize and protect RNA for isolation. The RNA stabilization solution also prevents collected teliospores from progressing to the next germination stage while collecting additional teliospores. Thirdly, it is important to remove the mineral oil once the collection droplet has been recovered to ensure successful RNA extraction. Lastly, we have noticed some loss of RNA quality if isolated teliospores are stored in RNA stabilization solution for an extended period of time; therefore, it is recommended that RNA is isolated immediately following collection of germination stage specific teliospores. A limitation of the method is that the Stage I teliospores collected could contain dormant, dead, and induced to germinate teliospores as these three stages are morphologically indistinguishable. In addition, when collecting Stage III teliospores, a mixture of teliospores in meiosis I or meiosis II could be obtained. One way to aid in distinguishing between truly dormant and dead teliospores could be to determine the viability of the sample. A method for assessing fungal spore viability using live/dead cell viability assays may be able to assess percentage of viable teliospores from which more informative germination rates could be determined[17]. In addition, nucleus staining with DAPI, for example, could be used to visualize the events of meiosis that are occurring during Stage III and the transition to Stage IV in order to further characterize teliospores morphologically at Stage III. This would aid in the collection of teliospores in only one stage of germination when using our microdissection method.

In conclusion, we have developed a simple, precise and reproducible method of measuring the changes in cellular respiration that occur during the dormancy-germination shift of *Ustilago maydis* teliospores. In addition, we have developed a method for collecting specific stages of germinating teliospores that could be used for downstream applications, such as RNA-seq. Our methods can be adapted to accommodate various cell-types and species. We anticipate that improvements to our techniques will facilitate the detection of respiratory changes on a single spore level as well as further defining the events that are occurring in the later stages of germination.

Disclosures

The authors have no competing financial interests or other conflicts of interest to disclose.

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