Please cite this article as: Jason and Armin , (2017). Isolation of *Ustilago bromivora* Strains from Infected Spikelets through Spore Recovery and Germination, *Bio-protocol* 7 (14): e2392. DOI: 10.21769/BioProtoc.2392.

bio-protocol www.bio-protocol.org/e2392 Vol 7, Iss 14, Jul 20, 2017
DOI:10.21769/BioProtoc.2392

Isolation of *Ustilago bromivora* Strains from Infected Spikelets through Spore Recovery and Germination

Jason Bosch and Armin Djamei*

Gregor Mendel Institute (GMI), Austrian Academy of Sciences, Vienna Biocenter (VBC), Dr. Bohr-Gasse 3, 1030 Vienna, Austria
*For correspondence: armin.djamei@gmi.oeaw.ac.at

[Abstract] *Ustilago bromivora* is a biotrophic smut fungus infecting *Brachypodium* sp. It is closely related to the barley-infecting smut *Ustilago hordei,* and related to the well-studied, gall-inducing model pathogen *Ustilago maydis.* Upon flowering, the spikelets of *U. bromivora*-infected plants are filled with black fungal spores. While it is possible to directly use this spore material to infect *Brachypodium* seeds, in many cases it is more useful to isolate individual strains of *U. bromivora* for a genetically homogenous population. This protocol describes how to collect and germinate the spores of *U. bromivora* on plate in order to obtain strains derived from a single cell.
Keywords: *Brachypodium distachyon*, *Ustilago bromivora*, Biotrophic interaction, Plant pathogen, Filamentous fungus, Head smut

[Background] *Ustilago maydis* infecting maize (*Zea mays*) has long been established as a model system for studying biotrophic pathogens (Brefort *et al.*, 2009). This has led to many discoveries concerning the nature of biotrophic interactions but has limitations due to the practical difficulties of working with maize in the laboratory. The same is true for the model fungus *Ustilago hordei* infecting barley (*Hordeum vulgare*) (Laurie *et al.,* 2012). In contrast to these crop plants, the model grass *Brachypodium distachyon* has a small genome, undemanding growth conditions and is amenable to genetic manipulation (Draper *et al.,* 2001). *B. distachyon* has also been used to study non-host resistance to *Puccinia striiformis* f. sp. *tritici* due to the genetic complexity of its usual host, wheat (An *et al.,* 2016). Recently, we have described *Ustilago bromivora*, a smut fungus related to *U. maydis,* which is able to infect *Brachypodium* sp. and proposed this as a new model system for studying biotrophic interactions (Rabe *et al*., 2016).

During infection of *Brachypodium* sp. by *U. bromivora,* no visible symptoms can be detected for most of the infection. The only visible symptom of infection occurs during flowering when the plant produces spikelets that are filled with black, fungal spores. These spores can be used to directly infect new seeds but contain genetically disparate fungal strains. For most purposes, a pure culture from a single cell is preferable as it can be cultured axenically, characterized and genetically manipulated before being used to infect further seeds. This protocol describes the process of germinating the *U. bromivora* spores *in vitro*. It bears similarities to spore germination protocols of other smut fungi, for example, *U. maydis* (Heinze, 2009; Nadal *et al*., 2016), but has been modified to account for differences in the host species and infected organs. Please be aware that *U. bromivora* shows a mating type bias leading to the survival

Please cite this article as: Jason and Armin , (2017). Isolation of *Ustilago bromivora* Strains from Infected Spikelets through Spore Recovery and Germination, *Bio-protocol* 7 (14): e2392. DOI: 10.21769/BioProtoc.2392.

of only one mating type (mat a) on plate after spore germination (Rabe *et al*., 2016). This means that it will require additional effort to generate the second mating type or the use of the strain which we have previously isolated.

Materials and Reagents

1. Paper bag (HERA, catalog number: 716P50)
2. 1.5 ml microcentrifuge tubes (SARSTEDT, catalog number: 72.690.001)
3. Petri dish (SARSTEDT, catalog number: 82.1473.001)
4. Micro-homogenizer (Carl Roth, catalog number: K994.1)
5. Pipetman Diamond tips, D200 (Gilson, catalog number: F161931)
6. Pipetman Diamond tips, D1000 (Gilson, catalog number: F161671)
7. Glass beads (Sigma-Aldrich, catalog number: 18406)
8. Copper sulfate ($CuSO_4$) (AppliChem, catalog number: 131270)
9. Ampicillin (Carl Roth, catalog number: K029.2)
10. Tetracycline (Duchefa Biochemie, catalog number: T0150)
11. Chloramphenicol (AppliChem, catalog number: A1806)
12. Potato dextrose broth (BD, catalog number: 254920)
13. Agar (BD, catalog number: 214040)
14. $PD_{Amp, Tet, ChlA}$ plates (see Recipes)

Equipment

1. Scissors
2. Cooled incubator (ST) ST 1 (Pol-Eko Aparatura, catalog number: ST 1)
3. Pipetman P1000 (Gilson, catalog number: F123602)
4. Pipetman P200 (Gilson, catalog number: F123601)
5. Vortex
6. Heraeus™ Pico™ 17 Microcentrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Pico™ 17, catalog number: 75002410)

Procedure

1. Harvest the infected spikelets from the plant by cutting them off using a pair of scissors. Figure 1 shows the appearance of the infected spikelets. Spikelets should be stored in a paper bag for ~10 days at 28 °C to dry out. They can then be stored at room temperature until use.

Please cite this article as: Jason and Armin , (2017). Isolation of *Ustilago bromivora* Strains from Infected Spikelets through Spore Recovery and Germination, *Bio-protocol* 7 (14): e2392. DOI: 10.21769/BioProtoc.2392.

Figure 1. Spikelets of *B. distachyon* infected by *U. bromivora*. The black masses of fungal spores are clearly visible in both the hydrated (right) and dehydrated (left) spikelet. The scale bar represents approximately 1 cm. The image background of the spikelets was digitally removed.

2. Carefully grind the spikelets filled with spore material with a micro-homogenizer in 1.5 ml microcentrifuge tubes to break up the spikelet. The spores are very robust to survive harsh environments and will not be damaged by the grinding.
3. Add 500 µl ddH_2O and continue to grind softly. It is best to use a rotating motion to grind the spikelet as pushing into the microcentrifuge tube will cause the liquid to splash out. The liquid will turn black as it is ground, indicating that the spores have been released and are properly suspended.
4. Incubate the ground spores in ddH_2O for 1 h at room temperature to allow faster-germinating, contaminating, fungal spores to germinate. This will leave them more susceptible to the sterilisation treatment and reduce overall contamination levels.
5. Add 500 µl 3% $CuSO_4$ and mix the solution either by pipetting up and down or by using a vortex. This will kill most, if not all, of the contaminating spores that have germinated but not the *U. bromivora* spores which can take up to 17 h to germinate.
   Note: $CuSO_4$ is a heavy metal and should be handled and discarded according to its MSDS.
6. Incubate the spore/$CuSO_4$ mixture for 15 min at room temperature.
7. Centrifuge the spore mixture at 1,200 *x g* for 5 min. This will cause the spores to pellet. Carefully pour out the liquid and re-suspend the spores in 1 ml ddH_2O.
8. Repeat step 7 three times but, on the third time, instead of re-suspending the spores in ddH_2O, proceed to step 9.
9. Re-suspend the spores in 300 µl ddH_2O with antibiotics (ampicillin, tetracycline and chloramphenicol). These will kill any non-fungal cells that might have survived the $CuSO_4$ treatment.

Please cite this article as: Jason and Armin , (2017). Isolation of *Ustilago bromivora* Strains from Infected Spikelets through Spore Recovery and Germination, *Bio-protocol* 7 (14): e2392. DOI: 10.21769/BioProtoc.2392.

10. Make a dilution series of the fungal spore suspension (10^0-10^{-4}) in ddH_2O with the three antibiotics.
11. Plate 100 µl of each dilution on a $PD_{Amp, Tet, ChlA}$ plate (see Recipes), spread using glass beads and incubate at 21 °C for several days to obtain colonies.
12. As *U. bromivora* spores contain tetrads, the original colonies should be singled out on PD plates (with or without antibiotics) to obtain colonies derived from a single cell.

Notes

While we have provided information on the specific equipment and reagents used, we have no reason to believe that it is essential to use them exactly. The equivalent equipment or reagents from other manufacturers should be just as suitable.

Antibiotic	**Final concentration** (µg/ml)
Ampicillin	100
Tetracycline	25
Chloramphenicol	25

Recipes

1. $PD_{Amp, Tet, ChlA}$ plates
   2.4% (weight/volume) potato dextrose broth
   2.0% (weight/volume) agar
   Note: Measure out the appropriate amount of potato dextrose broth and agar for the intended volume. Dissolve them in ddH_2O then autoclave the mixture at 121 °C for 15 min. Once it has cooled to approximately 50 °C, add ampicillin, tetracycline and chloramphenicol to their final concentrations. Pour the mixture into Petri dishes (20 ml per 9 cm Petri dish) and wait ~20 min for it to set.

Acknowledgments

This protocol was developed in the Djamei laboratory and has had aspects contributed and/or changed by multiple members of the Djamei group. Due to this, the people listed as authors are not the only ones who have been involved in developing the protocol (for this see Rabe *et al*., 2016) but are merely the ones who have prepared it for publication. The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement No. [EUP0012 'Effectomics'], the Austrian Science Fund (FWF): [P27429-B22, P27818-B22, I 3033-B22], and the Austrian Academy of Science (OEAW).

References

1. An, T., Cai, Y., Zhao, S., Zhou, J., Song, B., Bux, H. and Qi, X. (2016). *Brachypodium distachyon* T-DNA insertion lines: a model pathosystem to study nonhost resistance to wheat stripe rust. *Sci Rep* 6: 25510.
2. Brefort, T., Doehlemann, G., Mendoza-Mendoza, A., Reissmann, S., Djamei, A. and Kahmann, R. (2009). *Ustilago maydis* as a pathogen. *Annu Rev Phytopathol* 47: 423-445.
3. Draper, J., Mur, L. A., Jenkins, G., Ghosh-Biswas, G. C., Bablak, P., Hasterok, R. and Routledge, A. P. (2001). *Brachypodium distachyon*. A new model system for functional genomics in grasses. *Plant Physiol* 127(4): 1539-1555.
4. Heinze, B. (2009). Comparative analysis of the maize smut fungi *Ustilago maydis* and *Sporisorium reilianum*. *Philipps-Universitat Marburg*.
5. Laurie, J. D., Ali, S., Linning, R., Mannhaupt, G., Wong, P., Guldener, U., Munsterkotter, M., Moore, R., Kahmann, R., Bakkeren, G. and Schirawski, J. (2012). Genome comparison of barley and maize smut fungi reveals targeted loss of RNA silencing components and species-specific presence of transposable elements. *Plant Cell* 24(5): 1733-1745.
6. Nadal, M., Takach, J., Andrews, D., and Gold, S. (2016). Mating and progeny isolation in the corn smut fungus *Ustilago maydis*. *Bio Protoc*: e1793.
7. Rabe, F., Bosch, J., Stirnberg, A., Guse, T., Bauer, L., Seitner, D., Rabanal, F. A., Czedik-Eysenberg, A., Uhse, S., Bindics, J., Genenncher, B., Navarrete, F., Kellner, R., Ekker, H., Kumlehn, J., Vogel, J. P., Gordon, S. P., Marcel, T. C., Munsterkotter, M., Walter, M. C., Sieber, C. M., Mannhaupt, G., Guldener, U., Kahmann, R. and Djamei, A. (2016). A complete toolset for the study of *Ustilago bromivora* and *Brachypodium* sp. as a fungal-temperate grass pathosystem. *Elife* 5.