

***Ustilago maydis* Virulence Assays in Maize**

Amey Redkar^{1,3} and Gunther Doehlemann^{1,2*}

¹Max Planck Institute for Terrestrial Microbiology, Department of Organismic Interactions, Karl-von-Frisch, Germany; ²Botanical Institute and Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, BioCenter, Cologne, Germany; ³Current address: The Sainsbury Laboratory, Norwich Research Park, UK

*For Correspondence: g.doehlemann@uni-koeln.de

[Abstract] The basidiomycetous smut fungus *Ustilago maydis* (*U. maydis*) infects all aerial parts of its host plant maize (*Zea mays* L.). Infection symptoms are seen in the form of prominent tumors on all aerial parts of maize, after the establishment of a biotrophic interaction with the host usually around 5-6 days post infection (dpi). The fungus colonizes the various developmentally distinct aerial organs at different stages of development to form these prominent symptoms. Although being a biotrophic plant pathogen, *U. maydis* can easily be cultivated under axenic conditions to produce a standardized inoculum. The infections can be carried out under laboratory conditions by syringe inoculation on all the aerial organs of maize. This protocol has been successfully utilized to infect all the aerial organs of maize and formulate the virulence assays in *U. maydis* making it an excellent model system to study phyto-pathological investigations (Schilling *et al.*, 2014; Redkar *et al.*, 2015).

Materials and Reagents

1. 1 ml syringes (Henke Sass-Wolf, catalog number: 5100.200V0)
2. Needles (16 G, 40 mm) (Premier Healthcare & Hygiene, BD microlance, catalog number: 300637)
3. 50 ml Falcon tubes (Sigma-Aldrich, catalog number: T2318)
4. Glass test tubes (Carl Roth GmbH + Co, catalog number: HA79.1)
5. Soil (Frühstorfer Pikiererde T type soil) and Pots
6. *Ustilago maydis* strains

Note: The U. maydis wild-type or any transgenic strains are maintained as glycerol stock in -80 °C for long term storage. Every strain can be retrieved on a PDA plate.

7. Maize seeds (e.g. variety Early Golden Bantam or Gaspé Flint)

Note: The maize variety Early Golden Bantam is a standard maize line that requires around 4 weeks for the floral switch and development of the male inflorescence. The maize variety Gaspé Flint is a mutant maize that completes its life cycle in 35-38 days and hence has an early floral switch with the development of male inflorescence around 15 days after sowing. Hence this variety is suitable for floral infections with U. maydis.

8. Yeast extract (BD, Difco, catalog number: 210934)
9. Peptone (BD, Difco, catalog number: 211830)
10. Sucrose (Carl Roth GmbH + Co, catalog number: 9097.2)
11. Double distilled water
12. 2.4% Potato Dextrose Broth (BD, Difco, catalog number: 254920)
13. 2% agar (BD, Difco, catalog number: 214010)
14. YEPS_L medium (modified from Tsukada *et al.*, 1988) (see Recipes)
15. Potato Dextrose Agar (PDA) plates (see Recipes)

Equipment

1. Greenhouse
2. Erlenmeyer flasks (250 ml)
3. Disposable cuvettes (Carl Roth GmbH + Co, catalog number: Y195.1)
4. Incubator [e.g. Certomat BS-1 (B. Braun Biotech International)]
5. Centrifuge (Thermo Fisher Scientific)
6. Spectrophotometer [e.g. Ultrospec 3000 proUV/Visible (GE Healthcare)]
7. Knife

Procedure

A. Cultivation of maize plants

1. Maize varieties (that may have different lengths of developmental switch) (*E.g.* cv Early Golden Bantam or cv Gaspé Flint (or your maize cultivar of choice) are grown in round pots of 20 cm with 4 seeds in each pots at equal spacing. The pots are kept in a temperature controlled greenhouse with the following conditions (14-h/10-h light/dark cycle, 28/22 °C. Depending on the growth behavior of the plants, it must be ensured that the right stage is used for infection. Seedling infections are regularly done at the three leaf stage. Tassel infections are done when the tassel floral meristem is in the mitotic phase before the pollen formation (tassel size of 1-1.5 cm).
2. Both varieties can be grown in soil in approximately 15 cm round pots with 4 plants each. The variety Gaspé Flint can mainly be used for tassel (male inflorescence) infections as they have an early floral switch (15 days) as compared to the standard maize (early golden Bantam which is approx. 4 weeks).

B. *U. maydis* culture preparation

1. *U. maydis* strains growing on a fresh PDA plate (no older than 1 week) are used for preparation of pre-inoculum. The strains are inoculated in a 3 ml YEPS_L medium in a sterile glass test tube and incubated overnight at 28 °C with shaking at 200 rpm.

2. The pre-inoculum (40 μ l) is used for inoculation of the large media (50 ml) in a 250 ml sterile Erlenmeyer flask and then incubated at 28 °C with shaking at 200 rpm for around 10-12 h.
3. The cultures are then harvested when the optical density (OD₆₀₀) reaches to 0.6 to 0.8 (cultures should not be below 0.5, nor above 1.0). This range of OD ensures the growth of the *U. maydis* sporidia in the active dividing phase. The cultures are then centrifuged in 50 ml Falcon tubes at 900 x g for 5 min at room temperature.
4. The harvested cells are resuspended and washed in sterile water to remove all the media remains. The step B4 of centrifugation is repeated.
5. The cells are then resuspended by pipetting or vortexing in water to OD₆₀₀ = 1.0. This inoculum is used to infect the maize plants.

Note: For infection with wild type U. maydis strains (e.g. FB1 and FB2 which are the two compatible mating types) are to be mixed together in equal volume at OD₆₀₀ = 1.0 for a successful infection. In case of the solopathogenic strain SG200 (Kämper et al., 2006) or in case of any mutant resulting from this background one cultural strain can be used as inoculum.

C. *U. maydis* virulence assays

In seedlings:

1. Seedling infections are done with the maize plantlets that normally show three juvenile leaf stage. For most of the cultivars of maize the plants are 7 days old when this developmental stage is reached (Figure 1).

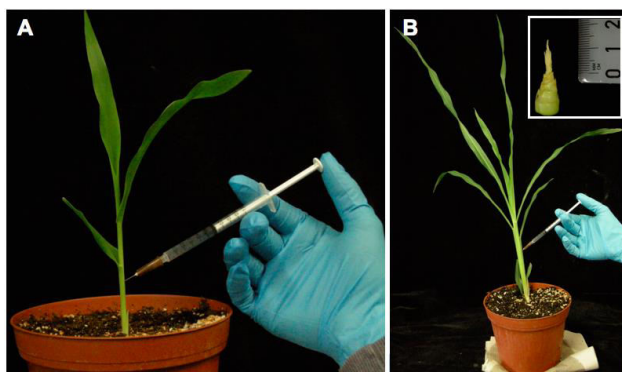


Figure 1. Maize plants cv. Early Golden Bantam and their inoculation with *U. maydis*. A. Maize seedling at the three leaf stage is inoculated with *U. maydis* for seedling leaf infection. The syringe points into the center of the leaf whirl. B. A maize plant is inoculated with *U. maydis* for tassel infection. The syringe hits the tassel inside the stem. Inset: the maize tassel at the stage of fungal infection.

2. The seeding infections are carried out by syringe with 300-500 μ l of the inoculum cell suspension into the interior of the leaf whorl. The injection site is chosen approx. 1 cm

above the soil, which is about 2.5 to 3 cm above the basal plant meristem and is the juvenile stem.

3. The leaf sheaths of the first and second leaf and the leaf blades of the third and fourth leaf which are immersed into the whorl are pierced by the syringe half way onto the center of the stem cylinder, which later show an infection mark after the symptom development.
4. Once the inoculum is seen on the inner whorl of leaves the seedling is known to be successfully infected.
5. Plants are kept in controlled growth conditions at 28/22 °C. Successful infection will lead to tumor appearance after 4-6 dpi.
6. The tumors formed by the artificial inoculation of seedlings are scored at 12 dpi by a modified standard scheme as described previously (Kämper *et al.*, 2006) which is shown in Table 1 and shown below (Figure 2).



Figure 2. Morphological symptoms in seedling (left) and tassel tissues (right) of maize after infection by *U. maydis*. Photographs were taken at 10 dpi.

Table 1. Classification of symptoms of infected maize seedlings

Plant symptoms	Description
No symptom	The plant shows no signs of infection
Chlorosis	The plant shows chlorotic discolouration of the infected leaves (third leaf and younger)
Small tumors	The largest tumors of the plant are <1.5 mm
Normal tumors	Tumors of the plant are 2-4 mm in diameter
Heavy Tumors	Very strong tumors leading to a stunted growth of stem
Dead Plant	The plant is dead and looks necrotic after infection with <i>U. maydis</i>

In tassels (male inflorescence):

1. Tassel infections are done immediately after floral switch of the plant when the tassel is still in the actively dividing mitotic phase. In cv. Gaspé Flint this is normally after 15 days and after around 4 weeks in cv. Early Golden Bantam after sowing (see Figure 1).
2. The tassel infections are also performed using a syringe based method. The immature tassels location is identified without cutting the plant by pressing the stem. The tassel meristem embedded in the stem has an air space surrounding it which makes the stem hollow. Above the tassel which terminates the shoot, the leaf sheaths overlap, but there is no solid center and this region contains the developing inflorescence.
3. Prior to injection, one or two plants are cut open with a sharp knife from the base, to understand the position of the tassel meristem that is embedded in the whorl of leaves.
4. Based on this height, the tassel location is approximately identified on all plants. The standard injection protocol involves inoculation of 1 ml of culture injected all at once, taking care to penetrate only halfway across the plant diameter. This ensures the inoculum to the entire floral meristem and fills the air space around the tassel.
5. Plants are kept in controlled greenhouse at 28 °C which show successful tumor appearance after 8-10 dpi (Figure 2).
6. Disease symptoms in the tassels are scored 10 days after infection in cv. Gaspé Flint and after 14 days in cv. Early Golden Bantam by the scoring scheme described previously (Schilling *et al.*, 2014, Table 2) and shown below.

Table 2. Classification of symptoms of infected maize tassels

Tassel Symptom	Description
Tumors<50% of tassel, small	The part of the tassel is converted into small tumors in range of 2-4 mm
Tumors<50% of tassel, large	Less than half of the tassel is converted into large tumors >4 mm
Tumors>50% of tassel, small	The entire tassel inflorescence is covered with small tumors.
Tumors>50% os tassel, large	The complete tassel is converted into heavy tumors by <i>U. maydis</i>
Tassel growth stunted	The tassel is arrested at the developmental time point at which it was infected and this 1-3 cm stunted tassel becomes tumorous.

Recipes

1. YEPS_L medium
This medium consists of 0.4% (w/v) yeast extract, 0.4% (w/v) peptone, 2% (w/v) sucrose in double distilled water.
2. PDA plates
The PDA medium consist of 2.4% Potato Dextrose Broth and 2% agar in double distilled water. After autoclaving approx. 18 ml of media in each petri plate.

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