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Running title:

Sporisorium reilianum virulence scoring protocol

Performing Infection Assays of *Sporisorium reilianum f. sp. Zeae* in Maize

Abstract

Corn head smut fungus *Sporisorium reilianum f. sp. Zeae* is a biotrophic pathogen belonging to the class of basidiomycetes. Under field conditions, it infects maize (*Zea mays L.*) still in the soil at early stages of development. Later, the infection spreads systemically to all aerial parts of the plant with mild symptoms of anthocyanin accumulation until the development of inflorescences, where it causes a replacement of maize inflorescences with spore-filled sori or leaf-like structures. Recently *Sporisorium reilianum* (*S. reilianum*) is being established as a model organism to study fungal-plant interactions and corresponding virulence factors. Here we describe a detailed protocol for a method that has been described and employed previously [1] to test the virulence of *S. reilianum* in maize under controlled laboratory conditions.

Key words *Sporisorium reilianum*, Maize disease, Infection symptoms, Scoring, Virulence, Head smut fungus

1 Introduction

Sporisorium reilianum is a biotrophic smut fungus and causative agent of head smut disease in maize (*S. reilianum f. sp. zeae*) and sorghum (*S. reilianum f. sp. reilianum*) [2], and is being established as a model organism to study fungal-plant interactions and corresponding virulence factors [3]. It is a close relative of *Ustilago maydis*, an intensive studied and well-established model organism, which infects the same host plant, *Zea mays* [4,5]. Although both smuts have a dimorphic life style i.e. a yeast like, haploid, saprophytic phase and a dikaryotic, filamentous, parasitic phase, they vary in their post-infection symptom development. The well-established

disease scoring protocol for *U. maydis* estimates mainly the severity of gall formation on all aerial parts of the plant as a measure of virulence. These galls are among the smuts rather exceptional symptoms and are visible already few days post infection of *U. maydis* in its host plant. In contrast to this, *S. reilianum* infection symptoms in maize can be mainly observed in the male (tassels) and female (ears) inflorescences of the plant where it causes a reversion of inflorescence into leaf-like structures (phyllody) or changes in kernel morphology ranging from elongated kernels to a substitution of kernels with white sori that contain the dark brown spores [3]. Moreover, *S. reilianum* infection leads to loss of apical dominance and an increase in female (ears) inflorescences [6], the complexity of the symptoms as well as the longer time period makes virulence scoring of *S. reilianum* a bit more challenging. This protocol is meant as a guide for interested students and plant pathologists to explore this fascinating pathosystem.

2 Materials

1. *S. reilianum* mating-type compatible strains *e.g.*, SRZ1 (5-2) and SRZ2 (5-1) [7] or solo-pathogenic strain *e.g.*, JS161 [5] (*see* **Note 1**)
2. Maize seeds (*e.g.*, Gaspe Flint) (*see* **Note 2**)
3. YEPSL medium, take 4 g of yeast extract, 4 g of peptone, and 20 g of sucrose and make up to 1L with double distilled water. Autoclave for 121 °C for 15 minutes.
4. Potato dextrose (PD) agar medium, take 24 g of Potato Dextrose Broth, 20 g of Bacto agar and make up to 1 L. Autoclave for 121 °C for 15 minutes, after autoclaving pour approximately 20 mL of media in each round Petri dish.
5. Double distilled water
6. Round Petri dishes (9.4 cm diameter)
7. Glass test tubes
8. Erlenmeyer flasks

9. Plastic pots of 20 cm diameter
10. T-type soil from Frühstorfer Pikiererde (*see* **Note 3**)
11. 1-mL syringes
12. Needles (16 G, 40 mm)
13. 50-mL Falcon tubes
14. Sterile bench
15. Rotary Shaker
16. Centrifuge with swing-out rotor
17. Temperature controlled climate chambers or greenhouse

3 Methods

3.1 Cultivation of Maize

1. Prepare 20 round pots of 20 cm diameter (19 cm height) with soil for each *S. reilianum* genotype you want to test.
2. Sow four seeds of appropriate maize variety e.g., Gaspe flint (*see* **Note 4**) 1 cm below the soil. After potting, soil should be soaked with water to ensure even humidity conditions important for seed germination. After germination, water moderately when soil gets dry.
3. Grow potted plants in a temperature-controlled greenhouse or if available climate chamber with the following conditions: 28 °C and 14 h of approximately 25,000 – 90,000 lux of illumination and 22 °C during the 10 h night period (*see* **Note 5**).
4. Infect seven days old maize seedlings with freshly in axenic culture grown *S. reilianum* cell suspensions by syringe infection (*see* **Note 6**).

3.2 Preparation of the S. reilianum Inoculum

1. For a pre-culture inoculation you need freshly on PD agar-plate grown colonies which should not be older than one week (*see* **Note 7**). You can use for maize infections either of the published *S. reilianum* solo-pathogenic strain [5] or two mating-type compatible strains of your choice [7] (*see* **Note 8**).
2. Inoculate 3 mL of YEPSL medium with a single fungal colony in a sterile glass test tube under a sterile bench and incubate overnight at 28 °C with constant agitation at 200 rpm. In case of the use of mating-type compatible strains, prepare two independent pre-cultures.
3. Next day, use the pre-culture to inoculate pre-warmed 50 mL of the YEPSL in a 250-mL sterile Erlenmeyer flask to an optical density at 600 nm (OD_{600}) of 0.1 cells. Grow further at 28 °C with constant agitation at 200 rpm until OD_{600} reaches 0.6 to 0.8 (*see* **Note 9**).
4. Harvest the cell cultures by centrifugation in 50-mL Falcon tubes at 900 x g in a swing out centrifuge for 10 minutes.
5. Discard the supernatant and re-suspend by pipetting up and down in 50 mL of sterile double distilled water the cell pellet. This step aims to remove all traces of YEPSL (*see* **Note 10**).
6. Pellet the washed cells by centrifugation at 900 x g in a swing out centrifuge for 10 minutes.
7. Repeat steps 5 and 6 and finally re-suspend the cell pellet in sterile water to an OD_{600} = 1.0 (in case of the solo-pathogenic strain) OD_{600} = 2.0 (for mating-type compatible strains) (*see* **Note 11**).

3.3 Infection of Maize Plants with S. reilianum

1. Inject 300-500 µl of cell suspension of *S. reilianum* strains into the leaf whorl of seven-day old maize seedlings with a syringe, approximately 2 cm above the soil.

   For this purpose, hold the syringe in an oblique position (Fig. 1) and pierce leaf sheaths of leaves in a way that the needle stays half way in the center of the stem cylinder. Do not push the needle through the stem (*see* **Note 12**). Press the syringe to inject the cell suspension until the inoculum is coming out from the upper end of whorl of leaves. [Fig 1 near here]
2. Grow plants further for seven weeks (*see* **Note 13**) under the same controlled growth conditions at 28 °C and 14 h of illumination and 22 °C during the 10 h night period.
3. Score disease symptoms seven weeks after infection (after inflorescence emergence) according to the procedure described in symptom classification.

3.4 Symptom Classification and Scoring

Disease symptoms of *S. reilianum* are scored as disease incidence (*see* **Note 14)** and a disease severity (*see* **Note 15)**.

1. For calculating **disease incidence,** place each injected plant in one of the nine categories described in 'categories of symptoms severity' column of Table 1 according to the severity of the symptoms based on each inflorescence (tassels and ears separately) (*see* **Note 16)**. [Table 1 near here]
2. Then calculate the percentage of plants in each category relative to the total number of inoculated plants (*see* **Note 17)** and these values can be plotted e.g., in an excel sheet as stacked column chart.

3. For calculating the **disease severity,** a comparable calculation as mentioned above can be performed. Collect all the inflorescences from all injected plants and sorted them by symptom category.
4. Next calculate the percentage of inflorescences in each category relative to the total number of inflorescences (*see* **Note 18)** and these values can be plotted e.g. in an excel sheet as stacked column chart. [Fig 2 near here]

4 Notes

1 *S. reilianum* mating-type or any transgenic strains are maintained as glycerol stocks at -80 °C for long term storage. Every strain can be retrieved on a PD agar plate at least three days before infection of maize plants.

2 *S. reilianum* causes symptoms mainly in reproductive organs of the maize plant, therefore the fast flowering and dwarfed maize variety Gaspe flint is routinely used for virulence assays as it has a short life cycle of around 8-10 weeks. However, any other maize variety can be used for this assay if respective greenhouse space is available but pot size and number of seeds per pot needs to be adapted according to the used variety.

3 T- type soil from Frühstorfer Pikiererde is heavily fertilized soil to be used for potting from spring to autumn. You can take any similar type of soil with high organic matter.

4 If you would like to test other varieties which are tall and late flowering *e.g.,* Early Golden Bantam, you need to sow only one seed per pot and take bigger pot size accordingly.

5 Maize seedlings need high temperatures 28 °C during the day and strong illumination, however if you do not have exactly above-mentioned light conditions, you can use 28 °C/22 °C and 14 h/10 h day and night cycles with the light conditions available to you but ideally minimum of 25,000 lux.

6 Seven days after potting, maize seedlings reach the three-leaf stage, inject the syringe from the site opposite of the third leaf and 2 cm above the soil half way into the stem.

7 Take your glycerol stock from the -80 °C freezer and transfer it to cooling block/rack. Take out a small smear of the glycerol stock by pocking it using a sterile loop or pipette tip under a sterile bench) then streak this sterile loop or pipette tip gently across the PD agar plate. Incubate the plate at 28 °C inverted for two days to obtain isolated colonies.

8 Transgenic strains should be grown on PD agar plates with appropriate antibiotics.

9 Optical density between 0.6 to 0.8 is essential to assure that the *S. reilianum* strains are in the exponential, actively dividing growth phase. It is therefore recommended to check the optical densities of the growing culture every 2 hours. The doubling time of *S. reilianum* is under axenic culture conditions at 28 °C around 2.5 hours [1].

10 Presence of sugars inhibit the pathogenicity and therefore infection.

11 Mix mating-type compatible strains (e.g. SRZ1 and SRZ2) in 1:1 ratio immediately prior plant inoculation. To reach OD_{600} = 1 of compatible pairs, the strains need to be resuspended to OD_{600} = 2 prior mixing.

12 If there is strong back pressure, it indicates that the needle is inserted into the stem and it needs to be retracted and reinserted a few millimeters higher on the plant.

13 Do not water the plants for 24 h after infection, as this is the period the fungus needs to attach to and penetrate its host.

14 The disease incidence defines the maximal damage induced by particular *S. reilianum* strain [1].

15 The disease severity provides information on how strongly an average plant is affected by the pathogen [1].

16 The categories represent the strongest symptom exhibited by the respective inflorescences, which means, an inflorescence may display symptoms of stronger and

weaker categories but only the strongest category is considered for that inflorescence. For example, if 5 % of the ear is replaced by sori and 95 % display elongated kernels (Fig. 2g), the ear belongs in the category 'less than 50 % spores' as spore formation is considered a stronger symptom than elongated kernels. A hierarchical order of symptom severity is following:

Spores > phyllody > healthy tassel

Spores > phyllody > leafy kernels > elongated kernels > immature ear > healthy ear

17 Percentage of plants in each category can be calculated, for example, if 6 out of 100 inoculated plants show tassels with spores, the percentage for this category would be 6 divided by 100 multiplied by 100 (6 %).

18 Number of total inflorescences evaluated differ from total number of plants as tassels and ears are considered separately for their morphological changes moreover, *S. reilianum* infection in maize results in increase in ear numbers [3].

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Table 1 Classification of symptoms of *S. reilianum* infected Maize plants

No.	Categories of symptoms severity
1	Tassel with spores (Fig 2c, 2d)
2	Tassel with phyllody (Fig 2e)
3	Ear with >50 % kernels filled with spores (Fig 2f)
4	Ear with <50 % kernels filled with spores (Fig 2g)
5	Ear developing >50 % leafy kernels (Fig 2h)
6	Ear developing <50 % leafy kernels (Fig 2I)
7	Ear developing elongated kernels (Fig 2j)
8	Immature ear, too small to detect disease symptoms (Fig 2k)
9	Healthy ear (Fig 2b)

Figure Legends

Fig. 1 Inoculation of maize plant (Gaspe flint) with *S. reilianum.* 7-day-old maize seedling being inoculated with *S. reilianum* mating-type compatible cell suspension using a syringe.

Fig. 2 Different symptom categories of *S. reilianum* infected maize inflorescences compared to healthy inflorescences. Morphology of (**a**) healthy tassel, (**b**) healthy Ear, (**c**, **d**) tassel with spores, (**e**) tassel with phyllody, (**f**) ear with > 50 % kernels filled with spores, (**g**) ear with < 50 % kernels filled with spores, (**h**) ear developing > 50 % leafy kernels, (**i**) ear developing < 50 % leafy kernels, (**j**) ear with elongated kernels, (**k**) immature ear. Scale bar = 1 cm.