

## Protocol

### Recovery, multiplication and infection typing of *Puccinia recondita* f. sp. *secalis*, causing leaf rust in rye (*Secale cereale* L.)

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#### Abbreviations:

DAI	Days after inoculation	Prs	<i>Puccinia recondita</i> f. sp. <i>secalis</i>
SPI	Single pustule isolate	RH	Relative humidity
IT	Infection type		

#### Overview

1. Collection of rust samples from field crops
2. Recovery and, multiplication of samples from field crops
3. Recovery and, multiplication of stock isolates from -80°C frozen liquid N<sub>2</sub>
4. Procedure for isolation and multiplication of single pustule isolates (SPI)
5. Large-scale inoculation using air brush spray system
6. Large-scale multiplication
7. Leaf rust phenotyping at seedling stage

## 1. Collection of rust samples from field crops

- Step 1:** Identify a green leaf with vigorous uredinia pustules and no other foliar diseases
- Step 2:** Detach leaf at basis using a scissor
- Step 3:** Tape the stretched leaf onto a clean piece of paper and fold to create a closed envelope
- Step 4:** Repeat above depending on number of samples required
- Step 5:** Dry the leaf segments (on paper) in a desiccator at least 24h and place weight on top to prevent leaf curling

## 2. Recovery and, multiplication of samples from field crops

Modified after Thach., *et al* (2015)

### 11 Days before inoculation:

- Step 1:** Sow 12-14 seeds of a susceptible cultivar or cultivar mixture at 1 cm depth in pots (7x7x8 cm) of Pindstrup Substrate peat mix containing slow-release plant nutrients (Pindstrup Mosebrug A/S, Ryomgaard, Denmark)

Note: Rye may be prone to establish poorly in heavy ('fine') soil types and if sown too deep (> 3-4 cm)

- Step 2:** Water the pot from above and place in a tray with sufficient water from below to cover 1/3 of the pot

- Step 3:** Place a lid on the tray to ensure 100% RH

- Step 4:** Cultivate under 16 hours of light at 18°C using a cultivation lamp (high-pressure sodium or LED) and 8 hours of dark at 12°C

**7-8 Days before inoculation:** Remove the lid at emergence of coleoptiles (2-3 cm plant height)

- 5 Days before inoculation:** Add 3.5 mL  $4.8 \times 10^{-3}$  M Antergon MH180 per pot, a growth regulator enhancing plant susceptibility to *Puccinia* spp. and increasing spore productivity

**Day of inoculation:** At half-emergence of 2<sup>nd</sup> leaf of seedlings

- Step 1:** Revitalize dried/fresh samples of infected rye, by placing leaf sections on a moist filter paper in a Petri dish, leave for 3-8 hours (dried samples) / up to 24h for fresh samples, at 100% RH until new urediniospores emerges

Note: If leaf segments are curled, place a clean object glass on top

- Step 2:** Carefully swipe seedling leaves in vertical direction for 10 seconds using presterilized gloves to 'break' the cuticula wax layer

- Step 3:** Inoculate the seedlings by repeatedly rubbing a revitalized leaf segment across the leaves in a horizontal direction to transfer the uredinio spores

- Step 4:** Carefully swipe the seedlings in a vertical direction to spread the spores uniformly

- Step 5:** Spray a fine mist of water across the seedlings from all angles

- Step 6:** Place the pot in a closed container with surface water to ensure 100% RH, and incubate at  $\approx 12.5^\circ\text{C}$  in dark for 24 hours

**1 DAI:** Transfer the multiplication pot to the greenhouse

Note: High humidity (>70% RH) during the latent period positively enhances the infection success

**7 DAI:** At first emergence of chlorotic spots on inoculated plants, cover each pot with a cellophane bag to prevent cross contamination by spores from neighboring pots (Figure 1A-B)

Note: Cellophane bags may delay/reduce rust spore production on rye. In case large amount of spores are required, it is recommend to multiply spores without cellophane bags, in single isolation cabins per isolate to avoid cross contamination. (See 'Large-scale multiplication' p. 7)

**Figure 1:** Multiplication of rye (*Secale cereale* L.) leaf rust, **A**) Emergence of chlorotic spots 7 days after inoculation, **B**) Pots placed in cellophane isolation bags to prevent cross-contamination.



**11-14 DAI:** 1<sup>st</sup> spore harvest

**Step 1:** Lift the multiplication pot slowly using the left hand, while putting a careful pressure to the seedling stalks, making a bent V shape so that loosened spores will collect in the cellophane bag (Figure 2A)

Note: The V-bent is to prevent soil from contaminating the spores when tapping the leaves

**Step 2:** Pot is laid down horizontally and transported to a flow cabinet

**Step 3:** Place a piece of weighing paper on the table, pre folded on the end to create a 'bottle neck'

**Step 4:** Cut the corner of the bag where spores are accumulated, and pour the spores onto the weighing paper by tapping the cellophane bag (Figure 2B)

**Step 5:** Remove the cut off cellophane bag corner using a pincer

**Step 6:** Transfer the spores to a pre-labeled tube

**Step 7:** Place the tube in a desiccator and loosen the lid, let dry for 3 days at 18-20°C

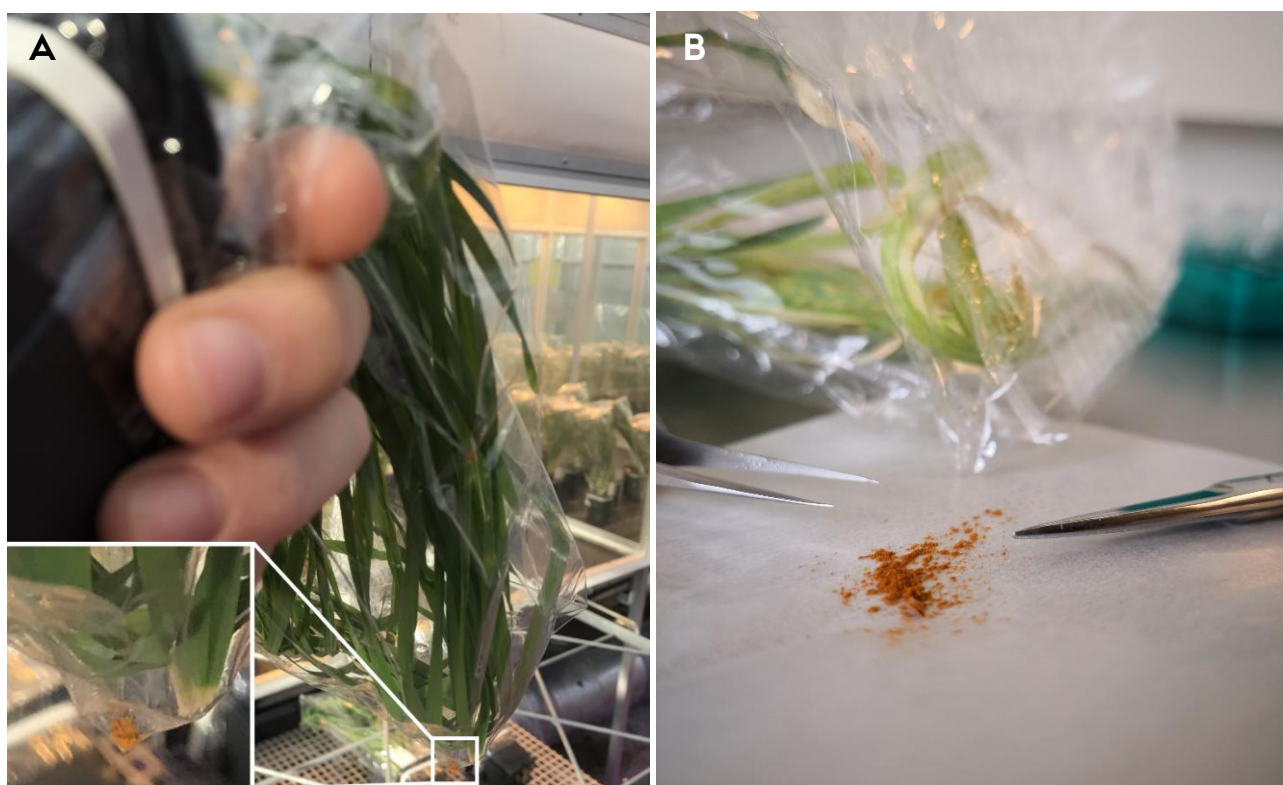
Note: Temperatures ( $\geq 25^{\circ}\text{C}$ ) may dramatically reduce spore vitality

**Step 8:** Place the tube in a  $-80^{\circ}\text{C}$  freezer for mid-long term storage

Note: Freezing of spores at  $-80^{\circ}\text{C}$  (or  $-20^{\circ}\text{C}$ ) may dramatically reduce spore vitality, in cases of, insufficient drying

**Step 9:** Repeat spore harvest 2-3 times until plant and rust vigor decreases

**Figure 2:** Multiplication of leaf rust spores on rye (*Secale cereale* L.), **A)** Harvest of spores 14 days after inoculation by tapping leaves, releasing spores to collect in the bottom corners, **B)** Spores poured onto weighing paper before transfer into tubes for mid-long term storage.



### 3. Recovery and, multiplication of stock isolates from $-80^{\circ}\text{C}$ frozen or liquid $\text{N}_2$

Modifications to above described protocol 'Multiplication of field samples'

**Day 0:** At half-emergence of 2<sup>nd</sup> leaf of seedlings

**Step 1:** Revitalize spores kept at  $-80^{\circ}\text{C}$  by heat shock at  $42^{\circ}\text{C}$  for 2 minutes

Note: Spores are stable at this stage for several days when kept at  $5^{\circ}\text{C}$  under dry conditions

**Step 3.1:** Pour the spores onto a piece of weighing paper

**Step 3.2:** Using presterilized gloves fixate the spores onto the index finger

**Step 3.3:** Inoculate the seedlings by stroking the leaves in a vertical direction for 30 seconds to transfer the uredinia spores and spread it uniformly

#### 4. Procedure for isolation and multiplication of single pustule isolates

As above with the following modifications: Single pustule isolates can either be isolated from field samples at low disease incidence or alternatively by low-density inoculation of seedlings in pots as described above.

**Figure 3:** Revitalization of rye (*Secale cereale* L.) leaf segment with leaf rust uredinia pustules. In this case, the fungus has expanded by fungal growth from the initial infection site on a seedling leaf, forming a circular ring of new pustules



#### 5. Large scale inoculation using air brush spray system

Modified after Thach., *et al* (2015)



**At time of inoculation** (half-emergence of 2<sup>nd</sup> leaf of seedlings)

**Step 1.1:** Pour spores into an air brush glass container

Note: If using spores from a frozen stock, revitalize as previously described

**Step 1.2:** Solubilize spores in 2 mL 3M<sup>TM</sup> Novec<sup>TM</sup> 7100 engineering fluid

**Step 3:** Inoculate the seedlings by spraying at 30-40 cm distance

Large-scale multiplication can be done using the two following procedures

1. Place support grids around the pots at 2 days after inoculation by gently 'forcing' the leaves through the upper ('second') grid window to get leaves hanging in a 45° angle



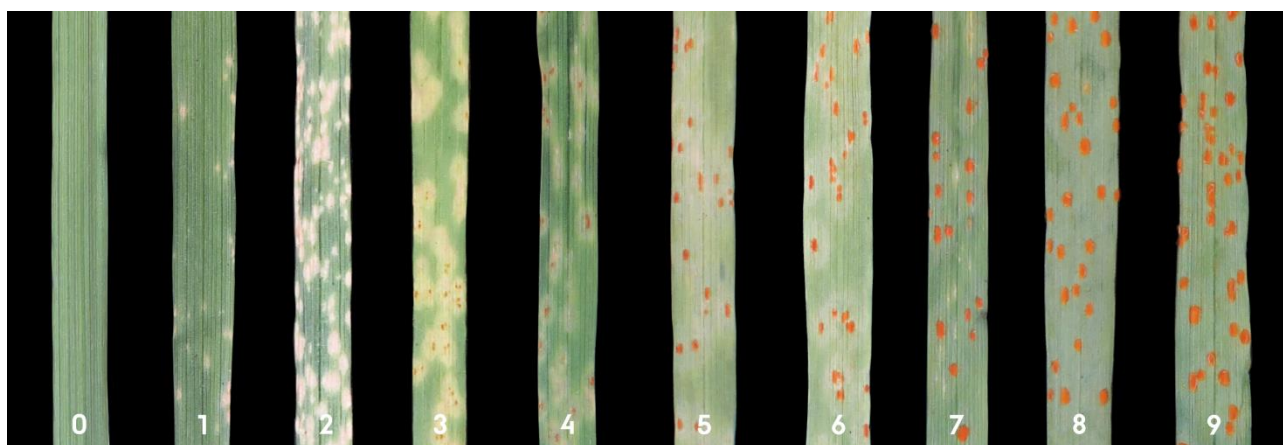
2. Place aluminum foil trays between each row in the tray 2 days after spray inoculation using air brush. At harvest, filter the spores through a sieve.



## 6. Leaf rust phenotyping at seedling stage

Seedlings are inoculated at the 2<sup>nd</sup> leaf stage allowing a scoring of infection types (IT) on both 1 and 2. Methodology for scoring of *Prs* SPI IT was adapted from Hovmøller, *et al.* (2017) about race typing of yellow rust in wheat. High-quality resolution picture of the ten IT in rye presented in (Figure 4), is available at <https://doi.org/10.5281/ZENODO.5478060>.

**Figure 4:** Infection type response (0-9) for leaf rust in rye (*Secale cereale* L.) caused by the fungal pathogen *Puccinia recondita* f. sp. *secalis* after Hovmøller, *et al.* (2017) and McNeal, *et al.* (1971). IT 0-2 are considered to represent ‘Resistant’, IT 3-4 ‘Partial resistant’, IT 5-6 ‘Partial susceptible’, and IT 7-9 ‘Susceptible’ host plants. In terms of virulence/avirulence, IT 0-6 are considered ‘avirulent’ and 7-9 ‘virulent’.



### References:

- Hovmøller, M. S., Rodriguez-Algaba, J., Thach, T., & Sørensen, C. K. (2017). Race typing of *Puccinia striiformis* on wheat. In *Wheat rust diseases* (pp. 29-40). Humana Press, New York, NY.
- McNeal, F. H., Konzak, C. F., Smith, E. P., Tate, W. S., & Russell, T. S. (1971). A uniform system for recording and processing cereal research data (No. REP-10904. CIMMYT.).
- Thach, T., Ali, S., Justesen, A. F., Rodriguez-Algaba, J., & Hovmøller, M. S. (2015). Recovery and virulence phenotyping of the historic ‘Stubbs collection’ of the yellow rust fungus *Puccinia striiformis* from wheat. *Annals of Applied Biology*, 167(3), 314-326.