Co-cultivation protocol for transforming wheat stripe rust

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Abstract

Protocol modified for *Puccinia striiformis* f. sp *tritici* (*Pst*), based on:

Michielse, C. B., Hooykaas, P. J., van den Hondel, C. A., & Ram, A. F. (2008). Agrobacterium-mediated transformation of the filamentous fungus Aspergillus awamori. *Nature protocols*, *3*(10), 1671-1678.

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Guidelines

Chemical solutions:

All chemicals used were from Sigma-Aldrich of the highest obtainable grades.

Buffers are best when fresh and not older than 3-6 months

- 0.2 M Acetosyringone (AS): Dissolve 785 mg of AS in DMSO to make up a total volume of 20 ml; aliquot and store in the dark at -20 °C. Do not thaw and use an aliquot more than twice, as activity of AS decreases during thawing and freezing
- 1% $CaCl_2$ (wt/vol): Dissolve 10 g of $CaCl_2 \cdot 2H_2O$ in water to make up a total volume of 1 litter; autoclave.
- 0.01% $FeSO_4$ (wt/vol): Dissolve 0.1 g of $FeSO_4 \cdot 7H_2O$ in water to make up a total volume of 1 liter; filter-sterilize.
- 20% glucose (wt/vol): Dissolve 200 g of glucose in water to make up a total volume of 1 litter; autoclave.
- 50% glycerol: Add 50 ml of glycerol to 50 ml of water to make up a total volume of 100 ml; autoclave.
- 1.25 M KH_2PO_4: Dissolve 170.1 g of KH_2PO_4 in water to make up a total volume of 1 litter; autoclave.
- 1.25 M K_2HPO_4: Dissolve 217.7 g of K_2HPO_4 in water to make up a total volume of 1 litter; autoclave.
- 1 M MES: Dissolve 195.24 g of MES in water to make up a total volume of 1 litter; adjust to pH

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5.5 by adding NaOH; filter-sterilize. Solution can be stored for a month in the dark or alternatively aliquot and frozen at -20°C.

- K-buffer: Add solution 1.25 M KH_2PO_4 to 1.25 M K_2HPO_4 until pH 4.8 is reached.
- MN buffer: Dissolve 30 g of $MgSO_4$•7H2O and 15 g of NaCl in water to make up a total volume of 1 litter; autoclave.
- 20% NH_4NO_3 (wt/vol): Dissolve 200 g of NH_4NO_3 in water to make up a total volume of 1 litter; autoclave.
- Trace elements for IM medium: Dissolve 100 mg of $ZnSO_4$•$7H_2O$, 100 mg of $CuSO_4$•$5H_2O$, 100 mg of H_3BO_3, 100 mg of $MnSO_4$•H_2O and 100 mg of Na_2MoO_4•$2H_2O$ in water to make up a total volume of 1 litter; autoclave.

Reagent set up:

- Hybond N+ filter (Amersham) need to be cut into small stripes at 5 mm in width. Do not autoclave.
- Induction medium (IM) (liquid): Add 0.8 ml of K-buffer, 20 ml of MN buffer, 1 ml of 1% (wt/vol) $CaCl_2$•$2H_2O$, 10 ml of 0.01% (wt/vol) $FeSO_4$, 5 ml of trace elements for IM medium, 2.5 ml of 20% (wt/vol) NH_4NO_3, 10 ml of 50% (vol/vol) glycerol, 40 ml of 1 M MES, pH 5.5, and 10 ml of 20% (wt/vol) glucose to 900.7 ml of sterilized water to make up 1 litter of liquid IM. No glucose IM just substitute 10 ml 20% glucose by 10 ml H_2O.
- IM (solid): Dissolve 15 g of bacto agar in water to make up a total volume of 905.7 ml; autoclave. Add 0.8 ml of K-buffer, 20 ml of MN buffer, 1 ml of 1% (wt/vol) $CaCl_2$•$2H_2O$, 10 ml of 0.01% (wt/vol) $FeSO_4$, 5 ml of trace elements for IM medium, 2.5 ml of 20% (wt/vol) NH_4NO_3, 10 ml of 50% (vol/vol) glycerol, 40 ml of 1 M MES, pH 5.5, and 5 ml of 20% (wt/vol) glucose to make up 1 litter of solid IM.

Wheat cultivar used: *Morocco*

A. tumefaciens strain: GV3101 pM90

Pst strain used: *P. striiformis* f.sp. *tritici* strain 104E137A-

Pst spore reproduction and collection:

Spores are resuspended in 3M™ Novec™ 7100 engineered fluids immediately before inoculation. 7-day old seedlings were inoculated with fresh urediniospores of *Pst* using flat brush and incubated for 24 h in 100% humidity at 8°C in the dark. Subsequently plants were transferred to a growth chamber at 17°C with a 16:8 light cycle. Spores can be harvested after 14 dai, with 2 days intervals, by dusting the heavily infected leaves on aluminium foil or a weighing paper.

GUS staining of _Pst_ spores:

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substrate sollution: 0.05M sodium phosphate buffer, pH7.4; 1mM EDTA; 0.1% (v/v) TritonX-100; 0.1mM $K_3Fe(CN)_6$; 0.5mg X-Gluc/ml

Freshly harvested *Pst* spores are germinated on the surface of water for 6 h, before replacing all water by the substrate sollution using glass Pasteur pipette. Spores in the substrate sollution are then incubated at 37 oC for 40 h, before moving to a glass slice using glass Pasteur pipette for imaging.

DNA extraction from *Pst* spores is according to Yiheng Hu: High quality DNA extraction from Fungi_small scale. protocols.io https://www.protocols.io/view/high-quality-dna-extraction-from-fungi-small-scale-exmbfk6

Critical steps to obtain high transformation efficiency:

- Must use fresh spores/wheat seedlings/Acetosyringone/*Agrobacterium*
- Must use glass plates/beakers to germinate spores
- Make sure the filter contain co-cultured spores attached tightly to the wheat leaf to maximize the infection.

Before start

1. Grow 12 pots of wheat seedlings (15 seeds/pot) and infect them with 50 uL of *Pst* spores to reproduce fresh spores for co-culture.
2. Plant certain numbers of wheat (depend on each experiment, I do 6 to 8 pots) 7 days before the experiment start (day 1)
3. Streak out *A. tumefaciens* contains the T-DNA in binary vector onto a LB-Agar plate and grow at 28°C for 48 h. Pick up one colony of the *A. tumefaciens* into 5 ml liquid LB media with proper selection and grow overnight. The co-culture experiment need to start from a nice overnight *A. tumefaciens* culture.

Protocol

Step 1.

Centrifuge the overnight *A. tumefaciens* culture at 5000xg for 10 mins, wash the pellet using induction media (IM, with glucose) once and resuspend the pellet with 5 mL IM (with glucose).

DURATION
00:10:00

Step 2.

Add 2ml of Agro-IM solution into 8ml liquid IM (with glucose) in a 50 mL falcon tube and put it at 28°C, shake until OD_{600}=0.6 (6 h depends on *Agrobacterium strain*)

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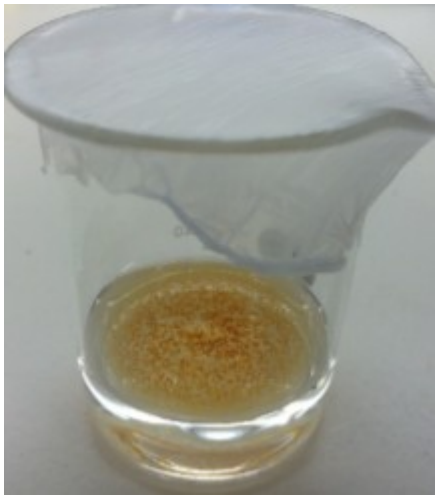
Step 3.

Harvest fresh spores from previous infected wheat, estimate the number of spores based on a counting experiment:

I first suspended them 0.1% (v/v) of the non-ionic detergent Triton X-100 to wet and separate them. Then I diluted the spore solution 10, 100 and 1000 times, and counted *Pst* spores using a haemocytometer I also estimated the spore volume by comparing the volume of a known number of spores with equivalent volumes of water in an Eppendorf tube. I estimated that 10 mg (20 ml) of *Pst* spores equals 3.5 x 10^6 spores.

Step 4.

Dust 100ul of spores onto a glass plate/beaker at 14 cm in diameter (indicated by the image below), gently spray water on to the surface using a water sprayer to help the spores distribute equally. Cover the plate using parafilm and put it into 8°C for 6 h



Step 5.

After the IM solution containing *A. tumefaciens* reaches OD=0.6, centrifuge at room temperature at 5000xg for 10mins, resuspend it using 10 ml liquid IM (without glucose) with 200 uM Acetosyringone (AS) added.

Step 6.

Take out four IM-Agar plates (with 200uM AS added) and take out the spores from 8°C. Using sterilized tweezers to tweeze the cut hybond N+ filter to attach spores from the surface. Dry the filter a little using the airflow in the hood for 1min, and put it onto IM-Agar (spore side up). Normally five pieces of filter strips per plate and two plates per pot of wheat seedlings (15 seedlings/pot).

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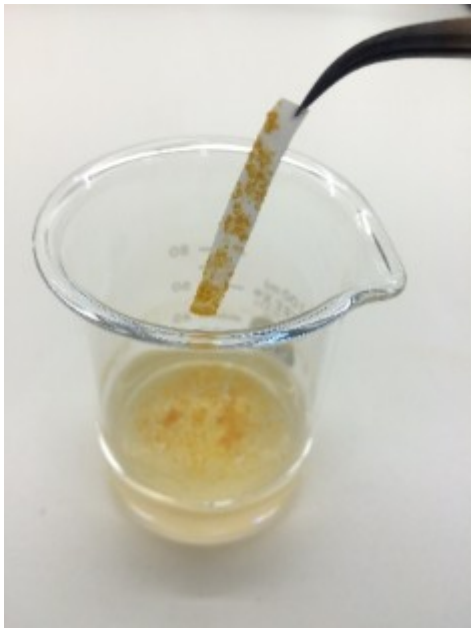
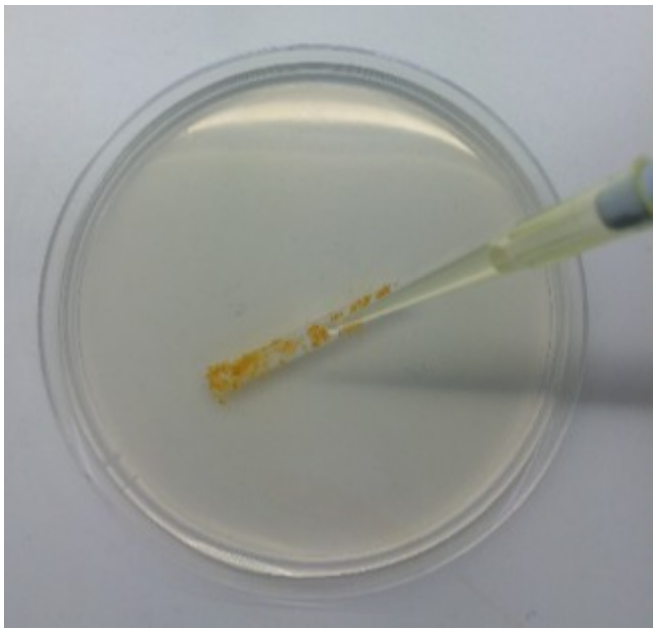


Step 7.

Pipette 100ul IM (no glucose, AS added) containing *A. tumefaciens* onto each filter with spores, wait them to dry for 3mins and put them at 20°C to process the co-culture.



DURATION

36:00:00

Step 8.

After co-cultivation of 36 h, take out the plates from the constant temperature room and prepare previously grown 6-8 pots of fresh 9-day old wheat seedlings. Tweeze the filter up from plate, gently spray water onto it and attach onto the wheat leaf (primary leaf). After all filter is attached, gently spray a little water onto the plants and then put them into 100% humidity (a container with water) at 8°C to germinate 24 h again.

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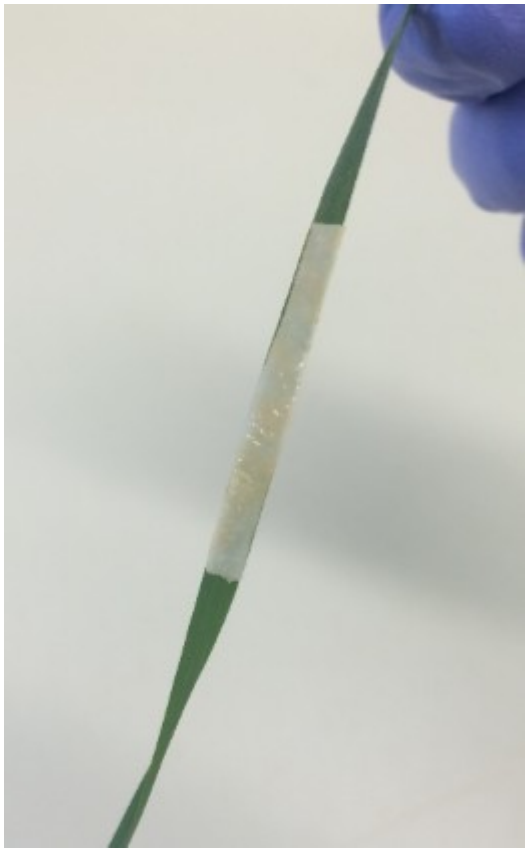

DURATION
24:00:00

Step 9.

Transfer the infected wheat into growth chamber (17°C 16:8 light:dark cycle, 60% humidity).

Step 10.

After 8 days, check the infection of spores on the wheat, and take off the filter papers to let pustules erupt.

Plant 8 pots of wheat to amplify the transformed spores for selection

Step 11.

Collect spores, reinfect a small number of spores for amplification and further analysis (GUS staining, DNA extraction etc.).

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