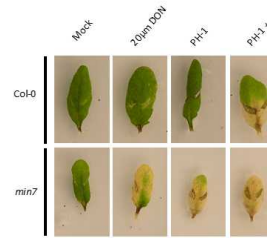




Mar 07, 2024

🌐 *Arabidopsis thaliana* detached leaf assay for the disease assessment of *Fusarium graminearum* infection

DOI

dx.doi.org/10.17504/protocols.io.261gedmwov47/v1Victoria J Armer^{1,2}, Michael J. Deeks², Kim E. Hammond-Kosack¹¹Rothamsted Research; ²University of Exeter

Victoria J Armer

Rothamsted Research, University of Exeter

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

[Create free account](#)OPEN  ACCESSDOI: <https://dx.doi.org/10.17504/protocols.io.261gedmwov47/v1>

Protocol Citation: Victoria J Armer, Michael J. Deeks, Kim E. Hammond-Kosack 2024. *Arabidopsis thaliana* detached leaf assay for the disease assessment of *Fusarium graminearum* infection. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.261gedmwov47/v1>

License: This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: February 05, 2024

Last Modified: March 07, 2024

Protocol Integer ID: 94706

Keywords: Fusarium graminearum, Arabidopsis thaliana, detached leaf, Deoxynivalenol (DON), fungal pathogen fusarium graminearum, infection arabidopsis thaliana, fusarium graminearum, leaf assay for the disease assessment, arabidopsis thaliana leaf, arabidopsis thaliana, fungal pathogen, detached leaf assay, pathogen, reproducible disease phenotypes for disease analysis, assessment of genotype, infection to the floral tissue, pathogen interaction, plant model organism, reproducible disease phenotype, diseases assessment, genotype, disease assessment, assessment of disease, disease analysis, genotypes in stock centre, fusarium graminearum infection arabidopsis thaliana

Funders Acknowledgements:

UK BBSRC South West Biosciences Doctoral Training Partnership

Grant ID: BB/T008741/1

UK BBSRC Delivering Sustainable Wheat Grant (DSW)

Grant ID: BB/X011003/1

UK BBSRC Delivering Resilience to Biotic Stress

Grant ID: BBS/E/RH/230001B

UK BBSRC Designing Future Wheat

Grant ID: BB/P016855/1

Abstract

Arabidopsis thaliana (*At*), as the plant model organism, has the advantage of a bank of genotypes in stock centres worldwide. This allows for the assessment of genotypes and their contributes to disease resistance and/or susceptibility to a wide range of pathogens. We found that, through utilising the SALK lines (Alonso *et al.*, 2003) provided by NASC (Nottingham, UK), to ensure reproducible disease phenotypes for disease analysis required additional specific steps not detailed in the current literature. This is likely due to the specificity of *Fusarium graminearum* (*Fg*) infection to the floral tissues of small grain cereals and the fact that *At* is a non-host of *Fg*. Whilst this protocol has been written and tested for the assessment of detached *At* leaves infected with *Fg*, it may be adapted for use for exploring other plant-pathogen interactions.

This protocol is designed for the assessment of disease on *Arabidopsis thaliana* leaves by the fungal pathogen *Fusarium graminearum*. The nuances of this protocol that have been troubleshoot have encouraged us to publish this as its own entity. The protocol described has been adapted from previous protocols published by Machado-Wood *et al.* (2021) and Chen *et al.* (2003), as well as by taking several components from Cuzick *et al.* (2008). Loosely, this protocol details seed sterilisation and plant growth, inoculations and diseases assessment.

Image Attribution

All images included in this protocol were taken by Victoria Armer, Rothamsted Research.

Materials

Protocol specific materials

1. *Arabidopsis thaliana* seed of desired genotype (NASC, UK)
2. Murashige and Skoog (MS) Basal Salt media (Sigma Aldrich, UK)
3. Deoxynivalenol (DON) (Sigma Aldrich, UK) *
**please read and follow the COSHH data sheet because DON is toxic to life.*
(<https://www.sigmaaldrich.com/GB/en/sds/sigma/d0156>).
4. Thin bleach (4.5% (v/v) Sodium hypochlorite)
5. Tergitol (Sigma Aldrich, UK)
6. 1M KOH

General lab consumables

1. 2ml microcentrifuge tubes
2. Toothpicks
3. dH₂O
4. Agar
5. Square petri dishes (120mm x 120mm)

General plant growth consumables

1. Levington's F2+S soil (Everris Ltd, UK)
2. Pots P7 (approx. volume 350cm³)
3. Plant trays
4. Capillary matting
5. Humidity lids

Equipment

1. Scales, weigh boats and spatulas
2. Stir bar
3. Duran flasks
4. Autoclave
5. pH meter
6. Fridge or cold room (5°C)
7. Flow hood
8. Controlled growth environment
8. Light meter

Troubleshooting



Safety warnings

- ⚠ Deoxynivalenol (DON) is a trichothecene mycotoxin that targets eukaryotic ribosomes, inhibiting protein translation. At chronic, low concentrations it can cause Leukaemia, and at higher, acute doses it induces nausea and vomiting, and can be fatal if swallowed. DON cannot be effectively destroyed by autoclaving. Please carefully read and follow the below COSHH data sheet and use appropriate PPE when handling DON.

(<https://www.sigmaaldrich.com/GB/en/sds/sigma/d0156>).

1.1. Preparation of ½ MS plates for *At* germination

- 1 *This step was found necessary to eliminate plate contamination from other fungal and bacterial species during seed germination on ½ MS agar plates. Unlike usual ½ MS preparations, this one does not contain sucrose to limit contamination.*


This can be done ahead of time and stored at room temperature for a matter of weeks after autoclaving.

- 1.1 Prepare ½ MS media using the following recipe (makes 1000ml – scale as required):


- 1.2 a.  1.1 g MS

- 1.3 b. Up to  1000 mL with dH₂O

- 1.4 c. Add stir bar and stir until suspended

- 1.5 d. Adjust to  5.7 with  1 Molarity (M) KOH

- 2 Aliquot into Duran flasks for autoclaving, removing stir bar





- 3 Add agar to  1 % (v/v) in each flask (e.g. 2.5g for 250ml) and autoclave before use

- 4 Pour ½ MS media into square plates to a depth of 25mm for seed germination.

1.2. Sterilisation and plating of *At* seed

- 5 *This was found to be the most ‘fool proof’ method of thorough seed surface sterilisation whilst ensuring high germination rates. N.B. don’t rush the serial dilution or miss steps – 5 washes ensures that the sterilisation solution is sufficiently diluted to not inhibit seed*



germination. Seed stratification in a cold room or fridge ensures even germination of At seed.

- 5.1 Create the sterilisation solution by mixing 50:50 dH₂O and thin bleach (4.5% (v/v) sodium hypochlorite). This must be made fresh on day of use.
- 5.2 Aliquot required seed into 2ml microcentrifuge tubes and add  500 µL of the sterilisation solution. Invert to mix, ensuring all seed are suspended before settling and leave for 10 mins, inverting several times to ensure sufficient coating of seeds.
- 5.3 Conduct a dilution series of the bleach solution, pipetting out  450 µL of the sterilisation solution and replacing with  1000 µL of dH₂O. Invert to suspend all seed and remove solution once seed settles. Repeat 4 times with subsequent additions of  1000 µL of dH₂O to ensure sufficient dilution of the sterilising solution.
- 5.4 Pour seed onto an absorbent surface (e.g. blue roll) for pricking out onto ½ MS plates, at least 20mm apart, using (autoclaved) toothpicks.
- 5.5 Place ½ MS plates into a plastic bag and place in the dark in a cold room or fridge for 48hrs.

1.3. Plant Growth

6 Plant growth

Plant growth can occur at neutral (12 hour) or long (16 hour) day length. We found that long day length worked well and sped up the experiment progression, allowing detached leaf inoculations to take place 4 weeks from germination.

- 6.1 Place square plates into a controlled growth room or cabinet with the following settings:
- 6.2 a. 16hr day length
- 6.3 b.  22 °C day temperature,  18 °C night temperature
- 6.4 c. 70% Humidity

6.5 d. $140\mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity.

6.6 Leave to germinate for 7 days.

7 Seedling transplantation

7.1 Prepare trays and pots for seed transplantation by lining trays with capillary matting and filling P7 pots with Levington's F2+S soil. Press until soil is firm and hydrate with dH_2O until moist to touch on the surface. Leave space (i.e. cut out one pot from the tray) for easy watering from beneath to avoid disturbing delicate seedlings.

7.2 At 7 days, transplant *At* seedlings from $\frac{1}{2}$ MS plates into F2+S using sterilised tweezers. Ensure roots are carefully tucked into the soil, pressing around the base of the developing rosette.

7.3 Spray the surface of the soil with dH_2O and place a vent closed humidity lid over the tray, leaving under normal growth conditions for a further 14 days (Fig. 1).

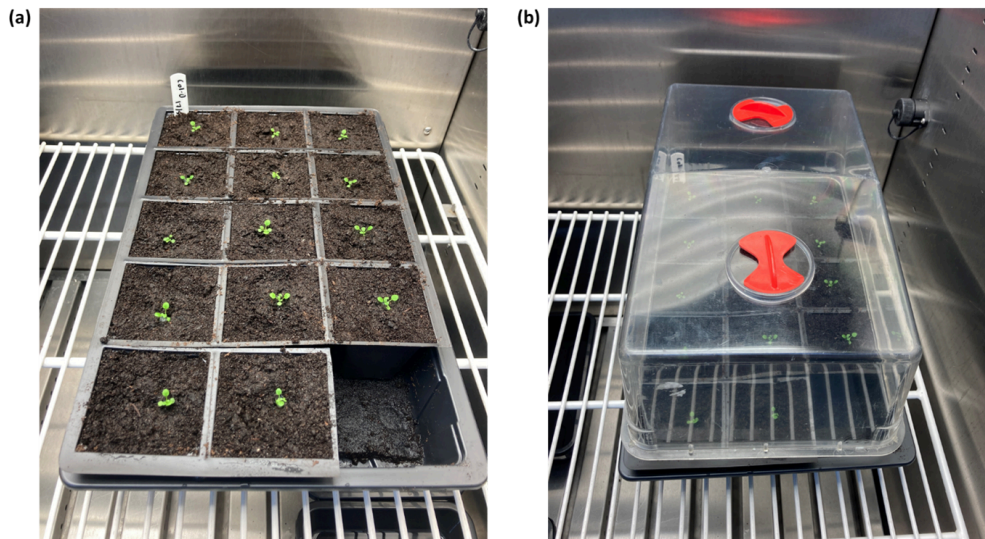


Fig. 1. Growth conditions of *Arabidopsis thaliana* in trays of P7 pots. Image of 21-day old seedlings. (a) Example growth tray lined with capillary matting and (b) with humidity lid (vent closed) for plant growth.

7.4 At 21 days old (14 days after transplantation) remove the humidity lid and leave for another 7 days. Ensure soil is well hydrated throughout plant growth, watering from the



bottom of the tray. Capillary matting should be moist to the touch and ensures even watering across the tray.

1.4. Detached leaf inoculations

8 *Detached leaf assay plates can be prepared a couple of days before inoculation if stored in a sealed bag in a cold room or fridge. The addition of Tergitol (a detergent and therefore fungal membrane stressor) encourages fungal growth on the At leaves rather than on the 1% water agar plates.*

8.1 Preparation of 1% (w/v) water agar plates for detached leaf assay:

8.2 a. Add  4 g Agar to a 400ml Duran bottle

8.3 b. Top up to  400 mL with dH₂O

8.4 c. Autoclave

8.5 d. Store at RT until use (can be prepared months in advance).

8.6 Melt 1% (w/v) water agar in the microwave (800W, 5.5 minutes)

8.7 Leave to cool until lukewarm

8.8 Add  200 µL Tergitol to each  400 mL 1% (w/v) bottle of water agar solution

8.9 Gently swirl flasks to disperse Tergitol without creating bubbles (about 20s of gentle motion)

8.10 Pour 1% (w/v) water agar plates to 25mm depth in flow hood and ensure plates are dry before use.

1.5. Rosette examples and leaf selection

- 9 At 4 weeks old, *At* plants should have well-developed vegetative growth but have minimal evidence of apical bud development (Fig. 2).



Fig. 2. Example rosettes of *At* wild-type Col-0 and susceptible control *min7* at 4 weeks under growth conditions described; scale bar = 20mm.

- 10 For the detached leaf assay, select leaves that are relatively small and flat for ease of inoculating, imaging and scoring. Leaves must be in active vegetative growth and not close to senescence – for easy identification, do not choose leaves that are particularly rounded indicating they originated in the early growth stages of the plant.
- 11 Cut leaves with sterile scissors and stick the petiole into the 1% (w/v) agar plate at a 45° angle. Ideally, leaves should not sit flush on the agar. Square plates can fit 6 leaves, spaced 30mm apart (Fig. 3).
- 12 Leaves should be selected and inoculated in quick succession i.e., leaves should be selected, cut and inoculated for each plate before moving onto the next plate.
- 13 Each plant is a biological replicate. Unless including technical replicates, only one leaf per plant is taken. The addition of Tergitol to the water agar prevents hyphal growth between replicates. The experiment should be repeated 3 times before conducting statistical analysis.

1.6. Inoculations

- 14 *The addition of DON is a requirement to make infection of *At* with *Fg* occur routinely. Efforts to achieve a reliable and reproducible assay without the application of DON have been unsuccessful.*

- 14.1 Selected leaves are gently scored on the agar plates using the end of a sterile 20 μ l (small) tip, either side of the midrib vein. This needs to be done very carefully to break through the adaxial epidermis layer, but not the palisade mesophyll layer. Several gentle strokes of the pipette tip may be required. This artificial wounding is necessary to allow *F. graminearum* to cause disease (Fig. 3.)



Fig. 3. Example square plate, filled with 1% water agar amended with Tergitol, laid with *Arabidopsis* WT Col-0 detached leaves. Wounding must damage the adaxial epidermis so that fungal infection can access the palisade mesophyll, but not penetrate the leaf entirely.

- 14.2 With gloved hands, drop 2 μ l of a 5×10^5 spores/ml water conidial suspension amended with 20 micromolar (μ M) DON onto each wound. Appropriate controls should be

used for each treatment to assess for the impact of scoring (dH₂O) and DON only application (20μM DON).

- 14.3 Plates should be placed in the dark for 2 days in otherwise normal growth conditions and subsequently left for a further 5 days under low light (70μmol m⁻² s⁻¹ light).

N.B. this can be achieved by moving plates further from light sources and under cover. Use a light meter to ensure that light levels are appropriate. Low light levels prevent senescence of leaves, which interferes with disease assessment.

- 14.4 Detached leaves should be assessed at 7 days post infection (dpi), although particularly strong phenotypes may be visible at 4dpi in susceptible genotypes.

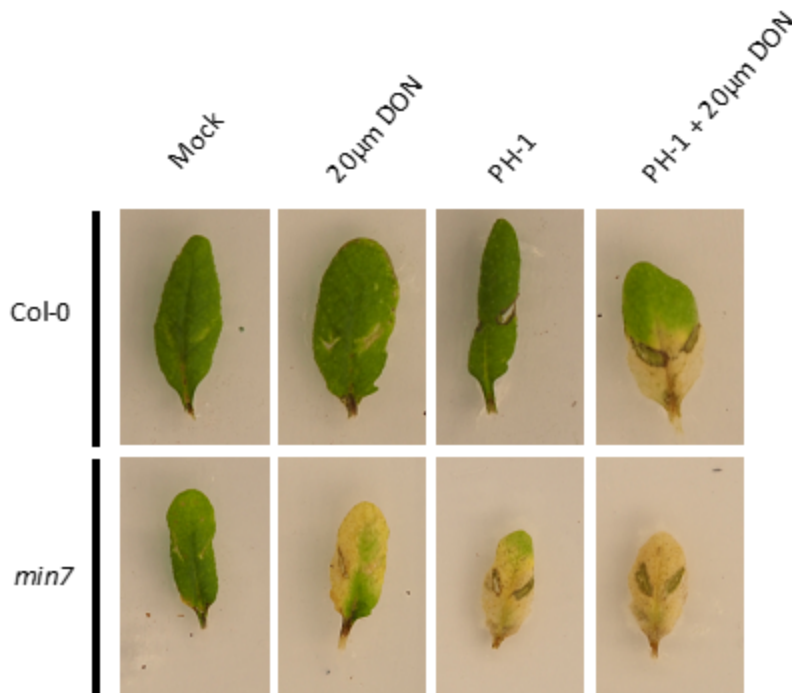


Fig.4. Example images of detached leaf assay at 7dpi for WT Col-0 and susceptible control *min7*.

14.5

Safety information

Deoxynivalenol (DON) is a trichothecene mycotoxin that targets eukaryotic ribosomes, inhibiting protein translation. At chronic, low concentrations it can cause Leukaemia, and at higher, acute doses it induces nausea and vomiting, and can be fatal if swallowed. DON cannot be effectively destroyed by autoclaving. Please carefully read and follow the below COSHH data sheet and use appropriate PPE when handling DON.

(<https://www.sigmaaldrich.com/GB/en/sds/sigma/d0156>).

Follow COSHH data sheet for the safe disposal of unused DON.

1.7. Disease assessment

15 **Image analysis**

Photographs of diseased images should be taken with a high-quality camera for recording purposes using a camera mount on a dark or light background.

16 **Visual scoring**

A DS index for visually scoring leaf disease severity is detailed by Chen *et al.* (2006) and offers a simple, reproducible way of assessing a large number of samples without downstream analysis.

17 **Fungal burden**

Fungal burden, the ratio of fungal to host gDNA, can be used to analyse the internal disease burden. A protocol for this is detailed in Machado-Wood *et al.* (2021), but briefly entails gDNA extraction followed by qPCR, with species-specific primers for quantification of entire gDNA.

18 **DON mycotoxin analysis**

The production of the DON mycotoxin by *Fusarium graminearum* can be measured as described in Cuzick, Urban and Hammond-Kosack (2008) using a commercially available enzyme-linked immunosorbent assay (ELISA). Ensure that the kit purchased matches the designated *Fg* chemotype of the strain to be used in the experiment; the WT reference strain PH-1 is a 15-ADON producer, an ester of deoxynivalenol (Miller, Taylor and Greenhalgh, 1983).

Protocol references

1. Alonso, J.M. *et al.* (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*. **301**(5633):653-7. doi: 10.1126/science.1086391
2. Chen, X., Steed, A., Harden, C. and Nicholson, P. (2006). Characterization of *Arabidopsis thaliana*-*Fusarium graminearum* interactions and identification of variation in resistance among ecotypes. *Mol. Plant Pathol.* **7**(5):391-403. doi: 10.1111/j.1364-3703.2006.00349.x
3. Cuzick, A., Urban, M., and Hammond-Kosack, K. (2008). *Fusarium graminearum* gene deletion mutants map1 and tri5 reveal similarities and differences in the pathogenicity requirements to cause disease on Arabidopsis and wheat floral tissue. *New Phytol.* **177**(4): 990-1000. doi: 10.1111/j.1469-8137.2007.02333.x
4. Machado-Wood, A. K., Panwar, V., Grimwade-Mann, M., Ashfield, T., Hammond-Kosack, K. E. and Kanyuka, K. (2021). The vesicular trafficking system component MIN7 is required for minimizing *Fusarium graminearum* infection. *J. Exp. Bot.* **72**(13):5010-5023. doi: 10.1093/jxb/erab170
5. Miller, J. D., Taylor, A., Greenhalgh, R. (1983). Production of deoxynivalenol and related compounds in liquid culture by *Fusarium graminearum*. *Can. J. Microbiol.* **29**(9): 1171-1178. doi: 10.1139/m83-179