Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

bio-protocol www.bio-protocol.org/e1877 Vol 6, Iss 14, Jul 20, 2016

Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues

Vamsi J Nalam[1], Sujon Sarowar[2] and Jyoti Shah[2,*]

[1]Department of Biology, Indiana University-Purdue University Fort Wayne, Fort Wayne, IN; [2]Department of Biological Sciences and BioDiscovery Institute, University of North Texas, Denton, TX
*For Correspondence: Shah@unt.edu

[Abstract] *Fusarium graminearum* (*Fg*) is the causal agent of Fusarium head blight disease of wheat (*Triticum aestivum*), oats (*Avena sativa*) and barley (*Hordeum vulgare*), which targets the floral tissues and thereby adversely impacts grain yield and quality. Mycotoxins produced by *F. graminearum* further limit the consumability of infected grain. In the laboratory, *F. graminearum* also has the ability to colonize both leaves and inflorescence tissues of *Arabidopsis thaliana*. The interaction between *A. thaliana* and *F. graminearum* makes available a large array of genetic and molecular tools to study the interaction between plants and *F. graminearum* to elucidate plant genes and pathways that contribute to resistance, as well as study how the fungus targets plant genes and mechanisms to promote disease. The methods described below allow for efficient infection of *Arabidopsis* leaves and inflorescence, and evaluation of disease progress and fungal growth. Disease spread in *Arabidopsis* can be readily monitored by the visual observations of chlorosis of leaf tissue and disease phenotype of inflorescence tissue including fungal mass on surface of the inflorescence tissue. Fungal growth can be further monitored by measuring the relative amount of *Fg* DNA in the host tissue by polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR).

Materials and Reagents

1. PCR tubes (Fisher Scientific, catalog number: 14222 262)
2. Petri dishes (100 x 15 mm) (Fisher Scientific, catalog number: FB0875713)
3. 50 ml plastic screw-capped tubes (Midsci, catalog number: C50B)
4. Pipette tips (sterile) (Midsci, catalog number: AVR-1, AVR-4 and AVR-11)
5. 1.7 ml microfuge tubes (sterile) (Catalog number: AVSS1700)
6. Cheesecloth from a local craft store or Miracloth (EMD Millipore, catalog number: 475855-1R)
7. Culture tube
8. 1 ml needle-less syringe (Tuberculin syringe) (Becton Dickinson, catalog number: 309659)
9. Funnel
10. 1 L glass conical flask (Pyrex brand)
11. Tweezers
12. Hemocytometer
13. Camel hair brush

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

14. Sharpie or comparable water-proof marker
15. Disposable gloves
16. Kimwipes, tissue paper or paper towels
17. Face shield (Fisher Scientific, catalog number: 18-999-4542)
18. Kord brand 3.5 inch square pots with bottom holes (Hummert International, catalog number: 12-1350-1)
19. T.O. Plastics Standard Flats 1020 tray with bottom holes (Hummert International, catalog number: 11-3000-1)
20. T.O. Plastics Standard Flats 1020 tray without holes (Hummert International, catalog number: 11-3050-1)
21. DOM1020 plastic dome to fit 1020 flats (Hummert International, catalog number: 11-3360-1)
22. Transparent plastic bags (Glad 13 gallon Recycling Drawstring Clear Trash bag)
23. *Fusarium graminearum* isolate Z-3639 (Bowden and Leslie, 1999)
24. *Arabidopsis thaliana* seeds (Accession Columbia, Nössen, and Wassilewskija)
25. Silwet L-77 (Lehle seeds, catalog number: VIS-30)
26. Potato Dextrose Broth (Becton Dickinson, catalog number: 254920)
27. Yeast extract (Becton Dickinson, catalog number: 212750)
28. BD Difco Agar (Becton Dickinson, catalog number: 214530)
29. Ammonium Nitrate (Fisher Scientific, catalog number: A676)
30. Potassium chloride (Fisher Scientific, catalog number: P217)
31. Magnesium sulfate heptahydrate (Fisher Scientific, catalog number: M63)
32. Sodium chloride (Fisher Scientific, catalog number: BP358-1)
33. Tris-Base (Fisher Scientific, catalog number: BP152)
34. Ethylenediaminetetraacetic acid, disodium salt, Dihydrate (Fisher Scientific, catalog number: S311)
35. Sodium dodecyl sulfate (Fisher Scientific, catalog number: BP166)
36. Carboxymethyl cellulose, CMC (Sigma-Aldrich, catalog number: C5678)
37. Sterile deionized water (dH_2O)
38. Sterile double distilled water (ddH_2O)
39. Phenol (Fisher Scientific, catalog number: BP226500)
40. Chloroform (Fisher Scientific, catalog number: C607-4)
41. Isopropanol (Fisher Scientific, catalog number: A451SK-4)
42. Ethanol (Fisher Scientific, catalog number: A995-4)
43. Primers (Listed below in Table 1)
44. dNTPs (Sigma-Aldrich, catalog number: DNTP100A-1KT)
45. Polymerase for PCR (Fisher Scientific, catalog number: FB-6000-10)
46. iTaq Univeral SYBR Green Supermix (Bio-Rad, catalog number: 1725122)
47. Agarose (Fisher Scientific, catalog number: BP1356)
48. Soil mix (Fafard, catalog number: Fafard Growing Mix 2/C-2)

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

49. Peters 20:20:20 General Purpose fertilizer (Hummert International; catalog number: 07-5400-1)
50. *F. graminearum* macroconidia suspension (see Procedure)
51. *F. graminearum* mycelial fragments (see Procedure)
52. Potato Dextrose Agar-Half strength (½ PDA) (see Recipes)
53. Carboxymethyl Cellulose (CMC) media (see Recipes)
54. *Arabidopsis* DNA extraction buffer (see Recipes)
55. Tris-equilibrated phenol-chloroform (see Recipes)
56. Spray spore suspension (see Recipes)

Equipment

1. Hand-held atomizer
2. Micropipettes (P20, P100 and P1000)
3. Standard Lab Incubator for cultivating fungus (Fisher Scientific, Fisher Scientific™ Isotemp™)
4. Plant growth chamber for cultivating *Arabidopsis* (Percival scientific, model: AR-66L2)
5. Thermal cycler (Techne, model: ^{3}PrimeX)
6. Real-time PCR system (Illumina, Eco™, catalog number: EC-101-1001)
   Note: This product has been discontinued by the manufacturer.
7. Compound microscope (Leica, model: DM2000)
8. Tabletop centrifuge (Beckman)
9. Microfuge (Fisher Scientific, Fisher Scientific™ accuSpin™, model: Micro 17/Micro 17R)
10. Vortex-Genie 2 (Scientific Industries, catalog number: SI-0236)
11. Basic power supply gel electrophoresis powerpack, trays and combs, (Bio-Rad, PowerPac™, catalog number: 1645050)
12. Gel electrophoresis system (Bio-Rad, catalog number: 1704405)

Software

1. Variance (ANOVA) ($P < 0.05$) (SAS Institute Inc, SAS v5.1)

Procedure

Experiments in our lab have shown that placing *F. graminearum* mycelia or macroconidia on the leaf surface does not yield uniform infection. Further, the level of infection is also highly variable. However, leaves when infiltrated with fungal mycelium fragments, which are small enough to enter the leaf tissue presumably through stomatal openings, resulted in reproducible infection. Macroconidia on the other hand are larger and could not be easily infiltrated into the leaves. On *Arabidopsis* floral tissue, macroconidia are able to germinate and successfully infect tissue.

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

A. Cultivation of *Fusarium graminearum* isolate Z-3639 and preparation of fungal mycelial suspension for inoculation of *Arabidopsis* leaves

1. The fungus is cultivated and maintained on ½ strength Potato Dextrose Agar (PDA) made with 0.7% Agar. Plates are 100 mm (wide) x 15 mm (deep).
2. To prepare fungi for inoculation, culture the *F. graminearum* isolate Z-3639 (Bowden and Leslie, 1999) on ½ PDA plates for 8-10 days at 28 °C. As the fungal mass grows, it turns a pinkish red color (Figure 1).

   Notes:

   a. *If a 28 °C incubator is not available the fungus can be cultivated at room temperature, although preferably not below 22 °C.*
   b. *Plates with fungal mass that are older than 1 month should not be used in the preparation of inoculum.*

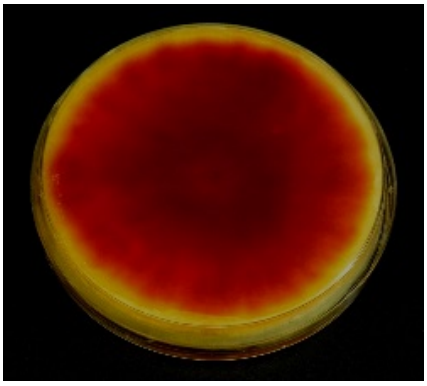

Figure 1. PDA plates showing red coloration due to *Fusarium graminearum* growth for 8 days at 28 °C

3. After 10 days, flood each plate with 10 ml of sterile ddH_2O, and carefully scrape mycelia from plate surface with a soft camel hair brush taking care not to scrape off the media (see Figure 2). This process harvests fungal mycelia from the media and simultaneously fragments it into smaller pieces, which is critical for the subsequent infection of *Arabidopsis* leaves. The fungal suspension will have a pink color to it (see Figure 2).

   Note: For control inoculations, use ½ PDA plates that were not inoculated with F. graminearum.
4. The fungal suspension is filtered through four layers of cheesecloth (alternatively can use two layers of miracloth) to remove debris and larger mycelial mass (see Figure 2). Finally, after suspensions from 6-7 plates are collected, pass 5 ml of sterile ddH_2O through the cheesecloth (or miracloth). Repeat this wash an additional time. Typically around 50 ml of suspension is required to infiltrate 60-70 leaves.

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

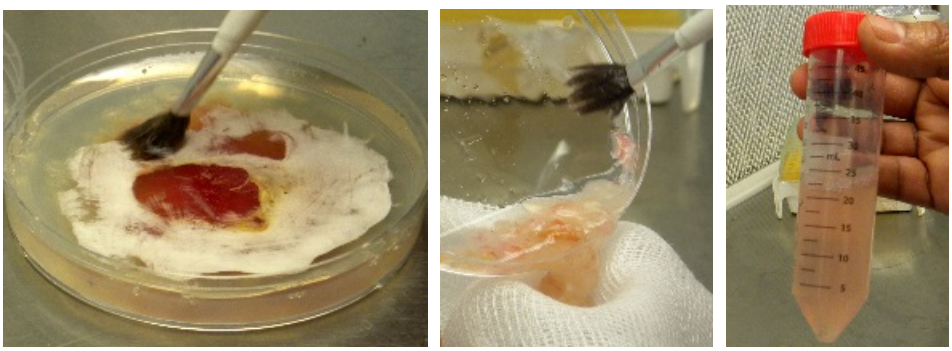

Figure 2. Preparation of fungal mycelial fragments from ½ PDA plates. Left panel: Harvesting fungal mycelia from ½ PDA plates with a camel hair brush. Middle panel: Filtering fungal mycelial suspension through cheesecloth. Right panel: Culture tube with filtered fungal mycelial suspension.

B. Cultivation of *Fusarium graminearum* isolate Z-3639 and preparation of fungal spores for inoculation of *Arabidopsis* floral tissues

1. Cultivate *F. graminearum on* ½ PDA plates for 8-10 days at 28 °C as described above.
2. To promote sporulation, a 1/4th square inch of fungal plug of fungal mycelial mass is cut from the PDA plate that shows profuse fungal growth and placed in a 1 L conical flask containing 250 ml of sterile carboxymethyl cellulose media.
3. Incubate the fungus-inoculated CMC media (see Recipes) containing flask on a shaker at 100 rpm at 28 °C for 7-9 days till profuse macroconidiation is observed.
4. The fungal suspension is filtered through four layers of cheesecloth to remove debris and mycelial mass.
5. The filtrate containing macroconidia is centrifuged in a table top swing-bucket centrifuge at 3,000 *x g* for 10 min.
6. The pelleted macroconidia are washed by re-suspending them in 10 ml sterile ddH_2O followed by centrifugation at 3,000 *x g* for 10 min at room temperature, as described above. This wash is repeated one more time.
7. The pelleted macroconidia (Figure 3) is re-suspended to a concentration of 1 x 10^5 macroconidia/ml in sterile ddH_2O water containing 0.001% Silwet L-77.

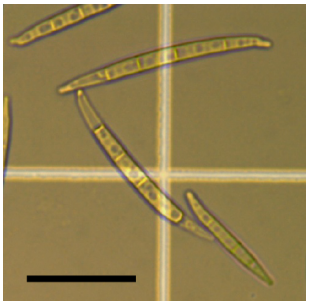

Figure 3. *Fusarium graminearum* macroconidia. Bar represents 20 µm.

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

C. *Arabidopsis* cultivation

1. A compost-peat-based Fafard #2 soil mix was used for cultivating *Arabidopsis*. The soil was first sterilized by autoclaving as follows: Soil sufficient to half-fill an autoclave bag is mixed with sufficient water to until complete saturation. At the same time, care must be taken to break large clumps of soil to ensure uniform soil saturation.
2. The loosely closed bag is autoclaved for 1 h. The soil is then allowed to cool to room temperature (overnight) before use.
3. The autoclaved, but cooled soil was loosely packed into Kord brand 3.5" square pots with bottom holes that were placed in 20-9/16" x 10-3/16" x 2-3/8" 1020 tray with bottom holes, which were further placed in 20-9/16" x 10-3/16" x 2-3/8" 1020 tray without holes (see Figure 4).

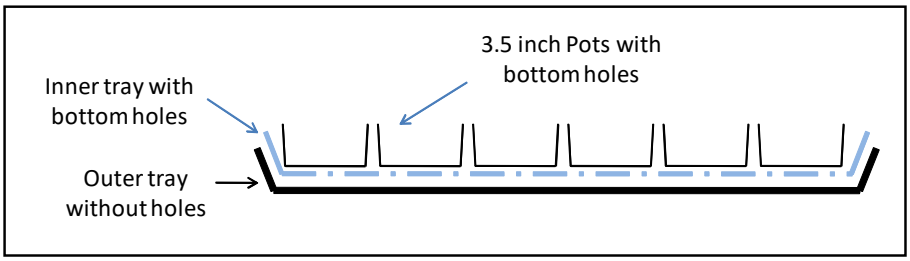

Figure 4. *Arabidopsis* cultivation set-up consisting of 3.5" pots contained in a tray with holes, which in turn is contained in a tray without holes

4. The soil was sub-irrigated by filling the outermost tray with tap water containing at 0.4 g/gallon of Peters 20:20:20 fertilizer and placing the soil-filled pots contained in the tray with bottom holes in it.
5. The soil was allowed to wet by capillary action till the soil surface was well wetted.
6. Excess water was drained by lifting the tray with bottom holes containing the pots above the water level.
7. The water in the flat without holes was drained off.
8. The drained pots in the flat with bottom holes were returned to the tray without holes.
9. Two seeds per pot were placed on the surface of the soil with a moistened toothpick, one seed at a time.
10. After all pots were seeded, the entire set up of pots in trays was covered with a transparent DOM1020 plastic dome and transferred into a cold room where they were left in the dark for stratification.
11. Two days later, the trays with the pots were moved into a growth room or growth chamber set at 22 °C under a 14 h light (80 μE m^{-2} sec^{-1})/10 h dark regime with approximately 60% relative humidity (RH).
12. Approximately four-week-old plants were used for inoculating leaf tissue with fungal mycelial fragments, while 6-7 week old plants with unbranched bolts were used for inoculating the inflorescence tissue with fungal macroconidia.

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

D. *Arabidopsis* leaf infection with *Fusarium graminearum*

1. Inoculation of *Arabidopsis* leaves with *Fusarium graminearum* mycelial fragments
   a. Approximately four-week-old *Arabidopsis* plants were used. It is important to include the appropriate control genotypes in each experiment. Plants are watered the day before inoculation. Infection is typically done in the afternoon hours.
   b. Expanded leaves for inoculation are marked with a water-proof marker. Approximately 4-5 leaves per plant are inoculated. A minimum of 60 leaves from 15 plants of each genotype are required for each treatment (mock v/s fungus).
   c. A 1 ml needle-less syringe is used for infiltrating a suspension of fungal mycelial fragments into the abaxial side (underside) of the *Arabidopsis* leaves (Figure 5). Leaves are infiltrated on each side of the mid-vein till the entire leaf area is infiltrated. Control (mock) treatment involves water that was passed over the PDA plates without the fungus and processed similarly to the processing of the fungal culture.
      Note: Use gloves, eye protection and lab coats when carrying out fungal inoculations. All waste coming in contact with the fungal culture is collected and autoclaved before disposing.
   d. After infiltration, plants are covered with a transparent dome for 48 h to maintain high humidity and promote fungal infection.

Figure 5. Fungal infiltration into *Arabidopsis* leaves. Shown is fungal culture being infiltrated with a needle-less syringe into the abaxial surface (undersurface) of an *Arabidopsis* leaf.

2. Scoring the severity of *Fusarium graminearum* disease on *Arabidopsis* leaves
   a. Disease spread is seen in the leaves as a spread of chlorosis and severity is recorded 5 days post inoculation. However, since disease progression depends on the quality of the fungal inoculum, if disease progression is slow then disease severity can be monitored at day 6 or even day 7.
   b. The percentage of inoculated leaves exhibiting chlorosis covering < 25% (category I), 25-50% (category II), 50-75% (category III) and > 75% (category IV) of leaf area are determined for each genotype (Figure 6). PCR analysis for fungal DNA relative to plant DNA is used to

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

confirm fungal growth over the course of infection (Figure 7 left panel) and determine correlation between disease severity and fungal growth in the diseased leaves (Figure 7 right panel).

c. A minimum of 60 leaves from 15 plants of each genotype are evaluated for disease severity.

d. Disease severity index is calculated using the formula

$$100 \sum \frac{(I\ n_I)}{(N\ k)}$$

Where,

I = disease severity score: 1 for category I, 2 for category II, 3 for category III and 4 for category IV,

n_I = number of leaves with each score,

N = total number of leaves,

k = highest score (in this case it is 4).

e. Single factor analysis of variance (ANOVA) ($P < 0.05$) (SAS v5.1) is used to compare disease severity amongst different genotypes. Figure 8 shows a representative data set comparing the disease severity between wild type (WT) plants of *Arabidopsis* accession Columbia (Col-0) and two mutants, *mut-1* and *mut-2*, that exhibit enhanced resistance.

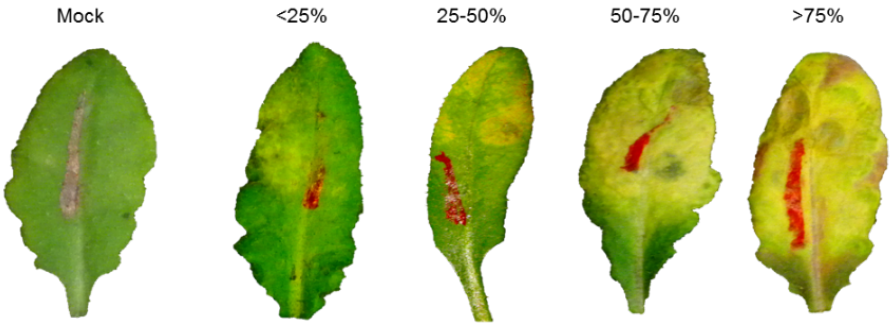

Figure 6. Disease symptoms in *Fusarium graminearum*-infected leaves. Diseased leaves are categorized into four groups based on the extent of leaf area exhibiting chlorosis.

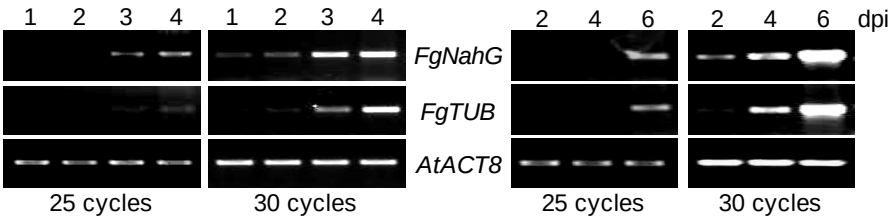

Figure 7. PCR analysis for fungal DNA. Left panel: PCR (25 and 30 cycles) for fungal and plant genes on DNA extracted from infected leaves exhibiting < 25% (lane 1), 25-50% (lane 2), 50-75% (lane 3), and > 75% (lane 4) chlorosis. Right panel: PCR (25 and 30 cycles) for fungal and plant genes on DNA extracted from 15 pooled leaves collected at 2, 4 and 6 days post

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

inoculation (dpi). PCR was conducted with primers specific for the fungal *NahG* (*FgNahG*) and *TUBULIN* (*FgTUB*) genes, and the *Arabidopsis ACT8* (*AtACT8*) gene (served as a control).

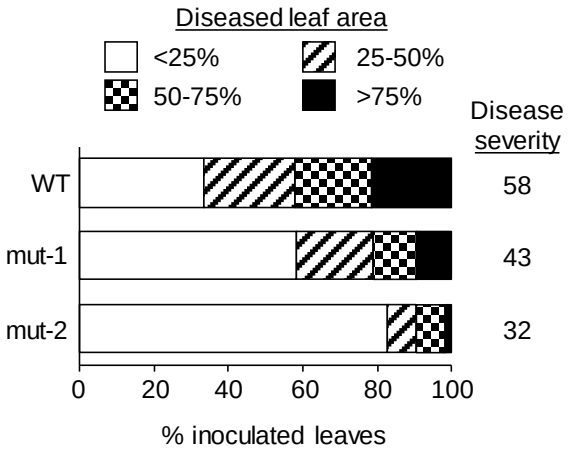

Figure 8. *Fusarium graminearum* disease severity in leaves of *Arabidopsis* wild-type accession Col-0 and two mutants (*mut-1* and *mut-2*) that exhibit reduced disease severity. The percentage of inoculated leaves exhibiting chlorosis covering < 25%, 25-50%, 50-75% and > 75% of the leaf area at 5 dpi was determined for each genotype. The disease severity index for each genotype is indicated on the right.

3. Monitoring fungal growth by PCR

   Infected leaf tissue is processed to extract DNA, which includes both, plant and fungal DNA.

   a. DNA extraction

      i. Fifteen fungus-inoculated and as control mock-inoculated leaves per *Arabidopsis* genotype are randomly harvested, pooled and quick frozen in liquid nitrogen.

      ii. The frozen tissue is ground to a powder in a chilled mortar with a chilled pestle. Approximately 50 mg of the frozen powder is transferred into a 1.7 ml microfuge tube containing 200 µl of DNA extraction buffer (see Recipes) at room temperature and mixed and left at room temperature for a minimum of 5 min to allow tissue dissociation.

      iii. Once all samples are processed, they are placed in a microfuge and centrifuged at 16,500 *x g* for 5 min to pellet cell debris.

      iv. The supernatant is transferred into a fresh 1.7 ml microfuge tube and mixed with 100 µl of Tris-equilibrated phenol-chloroform (pH 7-8) (see Recipes). Samples are centrifuged at 16,500 *x g* for 10 min at room temperature in a microfuge.

      v. The DNA containing supernatant is transferred into a fresh tube containing 150 µl of isopropanol. Vortex for 5 sec and leave samples at room temperature for 10 min.

      vi. Pellet DNA by centrifugation at 16,500 *x g* for 10 min at room temperature. Wash pellet with 500 µl 70% ethanol and then let pellet air dry left inverted on Kimwipes for 10 min.

      vii. Dissolve the pellet, which contains a mix of plant and fungal DNA in 200 µl of ddH_2O.

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

b. PCR for monitoring fungal growth in *Arabidopsis* leaves

i. Primers designed to the fungal genes *FgNahG* and *FgTUB* are used to monitor the amount of fungus relative to *Arabidopsis ACT8.*

ii. 1 µl of DNA extracted from plant tissue is used for PCR in a total 20 µl volume containing 0.25 µM each of dATP, dTTP, dCTP and dGTP, 0.05 µM of each primer, and 1 unit of Taq Pol (or related polymerase) along with the appropriate PCR buffer.

iii. PCR was conducted using the following amplification protocol: 3 min at 94 °C for denaturation of nucleic acids, followed by 25 or 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, culminating with a step at 72 °C for 30 sec and a hold step at 4 °C.

iv. The PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide and visualized under UV illumination (see Figure 7 as an example).

c. qPCR-based quantification of fungal growth in *Arabidopsis* leaves

Quantitative PCR (qPCR) for fungal genes (*FgNahG* and *FgTUB*) was performed with Sybr® Green PCR Master Mix on an Eco Illumina system (or any comparable real-time PCR machine) using the following amplification protocol: 10 min at 95 °C for polymerase activation and denaturation of nucleic acids, followed by 40 cycles of 95 °C for 10 sec, 58 °C for 30 sec and 72 °C for 30 sec. This was followed by a product melt to confirm a single PCR product. The level of gene expression was normalized to that of *Arabidopsis EF1α* by subtracting the C_T value of *EF1α* from the C_T value for the fungal gene. The $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) was used to calculate relative fold changes. See Figure 9 as an example.

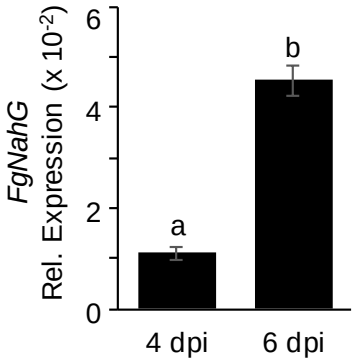

Figure 9. qPCR analysis to monitor fungal growth. qPCR analysis of fungal *FgNahG* gene relative to that of *Arabidopsis EF1*α conducted on DNA extracted from *Fg*-infected leaves at 4 and 6 dpi.

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

Table 1. PCR primers

Organism	Gene ID (name)	Primer Name	Primer sequence 5'→3'	Amplicon size	Application
Arabidopsis thaliana	AT1G07920 (*EF1 α*)	AtEF1α-F	TGCCGCAGGTGAATCAAAGG	113 bp	qPCR
		AtEF1α-R	CCCAATTACGAGAACAACGCTCTG		
Arabidopsis thaliana	AT1G49240 (*ACT8*)	AtACT8-F	AGCTCCGTATTGCTCCTGAA	419 bp	PCR
		AtACT8-R	TGGTTTTCGAGGTCTCCATC		
Fusarium graminearum	FGSG_06611 (*TUBULIN*)	FgTUB-F	TTGTCTCTGAACCAGCTCGT	496 bp	PCR
		FgTUB-R	AAGTTTGGACGTTGTTGGGG		
Fusarium graminearum	FGSG_08116 (*NahG*)	FgNahG-F	GGTGCTGATGGTATTCACTCT	156 bp	PCR and qPCR
		FgNahG-R	GTCGTGCATGAAGGTAGC		

E. Disease evaluation of *Arabidopsis* inflorescence tissue infected with *Fusarium graminearum*

1. Plant Inoculation

   a. Select flowering plants for inoculation *i.e.,* choose plants that possess an unbranched bolt with both open flowers on the terminal inflorescence and two to three developing siliques. It is important to include the appropriate control genotypes with each experiment.
   Note: *A minimum of ten plants should be chosen for inoculation. A similar number of control plants are sprayed with water.*

   b. With a permanent black marker, Mark the position on the flower stem above which only open flowers are present and below which siliques have begun developing.
   Note: *In order to minimize experimental error set up the plants in a randomized block design.*

   c. Spray spore suspension (1 x 10^5 spores/ml in sterile ddH_2O water containing 0.001% Silwet L-77) using a hand-held atomizer until droplet run-off has commenced.

   d. After this, re-inoculate each inflorescence with inoculum dispensed from the same sprayer (four sprays per flower head).

   e. Control plants are inoculated in the same way using de-ionized water containing 0.001% Silwet L-77.

   f. The inoculated plants were covered with a transparent plastic bag to ensure high humidity and placed in a plant growth chamber set at 22 °C under a 14 h light (80-100 μE m^{-2} sec^{-1})/10 h dark regime. Three days later the plastic bag was removed and the plants left in the growth chamber for an additional 4 days.

2. *Fusarium-Arabidopsis* Disease (FAD) score

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

a. Disease symptoms on individual inflorescence were monitored from day 3 onwards with the final disease score taken at 7 days post inoculation. However, if the progression of disease is rapid then the final disease score can be taken on day 5 or 6.
b. A numerical scoring system developed by Urban *et al*. (2002) (Table 2) was used to obtain the *Fusarium-Arabidopsis* Disease (FAD) score.
c. Disease phenotypes are assessed for three separate floral subcomponents (i) Flowers (F): infection covering open flowers and buds. (ii) New siliques (NS): Infection severity on siliques that developed after inoculation from flowers that were fully open at the time of inoculation. These flowers were located above the permanent mark placed on the stem at the time of fungal inoculation. (iii) Older siliques (OS): infection severity on siliques that existed at the time of fungal inoculation. For each of these components, the severity of infection is classified based on the macroscopic assessment of symptoms (Figure 10), as denoted in Table 2.
d. The final *Fusarium-Arabidopsis* disease (FAD) value is calculated by addition of the three subcomponent scores, *i.e.,* F + NS + OS = FAD as described by Urban *et al*. (2002). A representative data set is presented in Table 3.
e. *Arabidopsis* genotypes with FAD values of 3 and below are classified as exhibiting resistance to *Fg* whereas those with values of 10 and above are classified as susceptible.

Table 2. Classification of disease phenotypes and scores for flower, and new and old siliques. Adapted from Urban *et al.* (2002).

Organ	Score: Disease Phenotype
Flowers (F)	0: Normal looking flower with no obvious sign of disease
	1: Flower exhibits aerial fungal mycelium
	3: Flowers that have dried as a result of disease
	5: The stem within the flower head is constricted
New siliques[a] (NS)	0: Normal looking siliques with no obvious sign of disease
Older siliques[b] (OS)	1: Siliques with fungal mycelium on surface
	3: Siliques that have dried as a result of disease
	5: Constriction of the peduncle, or mycelia on peduncle, or loss of silique due to disease spreading beyond the silique
	7: Constriction of the main inflorescence stem due to disease progressing into the stem

[a]Siliques formed during the seven day period after fungal inoculation.

[b]Siliques that were present when plants were sprayed with fungal macroconidia

Please cite this article as: Vamsi *et. al.*, (2016). Establishment of a *Fusarium graminearum* Infection Model in *Arabidopsis thaliana* Leaves and Floral Tissues, *Bio-protocol* 6 (14): e1877. DOI: 10.21769/BioProtoc.1877.

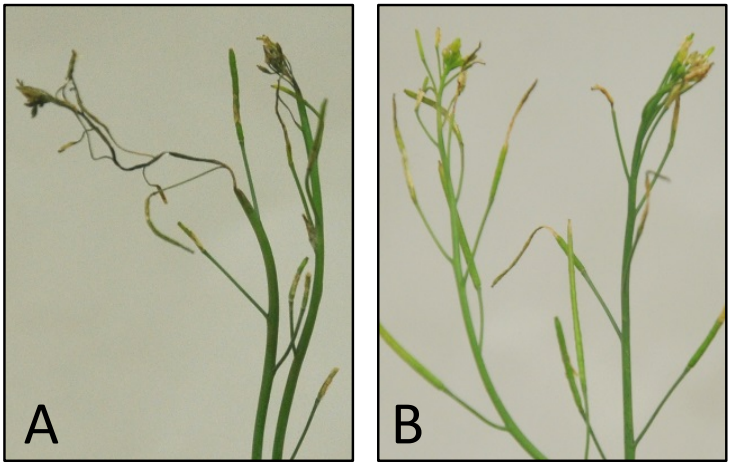

Figure 10. Symptoms of *Fusarium graminearum* disease in *Arabidopsis* inflorescence. A. A susceptible inflorescence showing disease symptoms. B. A relatively resistant inflorescence showing production of new flowers and siliques developed from flowers that were present seven days earlier at the time of fungal macroconidia inoculation.

Table 3. ***Fusarium graminearum* disease on inflorescence of *Arabidopsis* WT accession Col-0 and the *mut-1* and *mut-2* mutants**

Genotype	FAD
WT (Col-0)	6.3 ± 0.4
mut-1	1.9 ± 0.2
mut-2	1.8 ± 0.3

Recipes

1. Potato Dextrose Agar-Half strength (½ PDA)
   Potato dextrose broth powder 19.5 g
   Agar 7.0 g
   Distilled Water 1,000 ml
   Adjust pH to 5.6 ± 0.2 at 25 °C, prior to adding Agar.
   Sterilize by autoclaving for 20 min. Pour and allow to set approximately 30 ml into each 100 x 15 mm petri dish.
2. Carboxymethyl Cellulose (CMC) media
   NH_4NO_3 1.0 g
   KCl 0.2 g
   $MgSO_4 \cdot 7H_2O$ 1.0 g
   Yeast extract 1.0 g
   Carboxymethyl cellulose 26.0 g
   Distilled water to 1,000 ml

Sterilize by autoclaving for 20 min.

3. *Arabidopsis* DNA extraction buffer
   200 mM Tris-Cl, pH 7.5 (Sambrook *et al.*, 1989)
   250 mM NaCl
   25 mM EDTA, pH 7.5 (Sambrook *et al*., 1989)
   0.5% SDS
4. Tris-equilibrated phenol-chloroform (Sambrook *et al*., 1989)
5. Spray spore suspension
   1 x 10^5 spores/ml in sterile ddH_2O water containing 0.001% Silwet L-77

Acknowledgments

This work was supported by funding from: the U.S. Department of Agriculture (Agreement #59-0200-3-003 and 59-0790-8-060) as cooperative projects with the U.S. Wheat & Barley Scab Initiative. The protocols described here are based on the procedures developed and described by Makandar *et al*. (2010), Nalam *et al*. (2015) and Urban *et al*. (2002).

References

1. Bowden, R. L. and Leslie, J. F. (1999). Sexual Recombination in *Gibberella zeae*. *Phytopathology* 89(2): 182-188.
2. Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4): 402-408.
3. Makandar, R., Nalam, V., Chaturvedi, R., Jeannotte, R., Sparks, A. A. and Shah, J. (2010). Involvement of salicylate and jasmonate signaling pathways in *Arabidopsis* interaction with *Fusarium graminearum*. *Mol Plant Microbe Interact* 23(7): 861-870.
4. Nalam, V. J., Alam, S., Keereetaweep, J., Venables, B., Burdan, D., Lee, H., Trick, H. N., Sarowar, S., Makandar, R. and Shah, J. (2015). Facilitation of *Fusarium graminearum* infection by 9-Lipoxygenases in *Arabidopsis* and Wheat. *Mol Plant Microbe Interact* 28(10): 1142-1152.
5. Sambrook, J., Fritch, E. F. and Maniatis, T. (1989). Molecular Cloning: a laboratory manual. *Cold Spring Harbor Laboratory*.
6. Urban, M., Daniels, S., Mott, E., and Hammond-Kosack, K. (2002). *Arabidopsis* is susceptible to the cereal ear blight fungal pathogens *Fusarium graminearum* and *Fusarium culmorum*. *Plant J* 32: 961-973.

