

Airbrush Infiltration Method for *Pseudomonas syringae* Infection Assays in Soybean

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[Abstract] We developed this protocol to assay the extent of proliferation of *Pseudomonas syringae* pv. *glycinea* in soybean leaves. This method specifically enables accurate pathogenesis assays of soybean plants at V2/V3 (2nd/3rd trifoliolate) or higher stages of growth. The leaves of soybean plants at these growth stages are not amenable to bacterial infiltration using routine needleless syringe infiltration due to the high number of trichomes on these mature leaves. This method enables efficient infiltration of bacteria into the epidermal cells of mature leaves using a pressure pump.

Materials and Reagents

A. Plant material

Soybean (*Glycine max* Merr.) plants of V2 (2nd trifoliolate), V3 (3rd trifoliolate), or higher stages of growth were used. Plants at VC (two leaf stage)/V1 (1st trifoliolate) stages can also be used. Cultivars for various *R* loci: *Rpg1-b* (Harosoy), *Rpg2* (Merit, Norchief), *Rpg3* (Flambeau), *Rpg4* (Flambeau), *rpg* (Essex).

B. Bacterial strains

1. *P. syringae* pv. *glycinea* expressing *avr* gene of interest via the broad host range plasmids pDSK519 or pDSK600.
2. *P. syringae* pv. *glycinea* expressing empty pDSK519/600 plasmids as control.

C. Media and buffers

1. 10 mM MgCl₂ (sterile)
2. King's B medium (plates and liquid media) (see Recipes)

D. Other reagents

1. Tryptone (Teknova, catalog number: T9012)
2. K₂HPO₄ (Fisher BioReagents, catalog number: BP363)
3. Glycerol (Affymetrix, catalog number: 16374)
4. Agar (Affymetrix, catalog number: 10654)

5. Silwet L-77 (Momentum, New Smyrna Beach)
6. Antibiotics (Gold Biotechnology)
 - a. Rifampicin (50 mg/ml) (R-120)
 - b. Kanamycin (50 mg/ml) (K-120)
 - c. Spectinomycin (100 mg/ml) (S-140)
 - d. Streptomycin (300 mg/ml) (S150)

Equipment

1. High-speed floor centrifuge (Thermo Fisher Scientific, Sorvall RC 6 plus)
2. Spectrophotometer (Thermo Fisher Scientific, model: BioMate 5)
3. Pressure pump (Gast Manufacturing, model: DOA-P704-AA)
4. Airbrush (Badger Air-brush, model: 250-2)
5. Test tubes (Thermo Fisher Scientific)
6. Microcentrifuge tubes (Thermo Fisher Scientific)
7. Glass rods (Thermo Fisher Scientific)
8. Pellet pestles (Sigma-Aldrich)
9. Cork borer (1 cm diameter, Thermo Fisher Scientific)

Procedure

A. Bacterial growth and inoculum preparation

1. Streak -80 °C stock of bacterial culture on King's B agar with required antibiotics (Rifampicin and Kanamycin for pDSK519 plasmid; Rifampicin, Spectinomycin and Streptomycin for pDSK600 plasmid). Incubate the plate at 29 °C till bacterial colonies grow (24-48 h).
2. Inoculate a single colony of *P. syringae* in 10 ml King's B medium with required antibiotics (Rifampicin and Kanamycin for pDSK519 plasmid; Rifampicin, Spectinomycin and Streptomycin for pDSK600 plasmid).
3. Grow 12-16 h at 29 °C on rotary shaker at 200 rpm until the culture reaches OD (A_{600}) 0.8-1.2. Do not use culture at $OD_{600} < 0.8$, or > 1.2 .
4. Centrifuge culture at 3000 rpm for 10 min. Discard the supernatant and resuspend the bacterial pellet in 10 ml, 10 mM $MgCl_2$ by gentle pipetting (do not vortex).
5. Measure OD (A_{600}) of the bacterial suspension (this should be the same OD as your culture from King's B medium, 0.8-1.2) and dilute to 10^5 CFU (colony forming units)/ml using 10 mM $MgCl_2$ (1.0 OD measurement approximately corresponds to 2×10^8 CFU/ml).
6. Add Silwet L-77 to a final concentration of 0.01% to the bacterial suspension and mix gently. Use bacterial suspensions within 1 h after preparation.

B. Soybean infiltration

1. Attach appropriate ports of airbrush (Figure 1) to the pressure pump (40 psi or less, Figure 2) and beaker containing bacterial suspension using rubber tubing.
2. Infiltrate soybean plants at V2 stage on the abaxial surface of the trifoliate leaves using the airbrush, while holding the leaf against a flat surface (such as a Petri plate) ensuring that the pressure does not damage the leaf during infiltration (see Video 1).
3. Inoculate one trifoliate per plant, and at least 5 plants per bacterial strain. 5-8 ml of bacterial inoculum is sufficient to completely infiltrate one trifoliate.
4. Mock inoculations should be done similarly 10 mM MgCl₂ + 0.01% Silwett L-77 instead of the bacterial suspension.



Figure 1. Image of airbrush used for bacterial infiltration



Figure 2. Image of pressure pump used for bacterial infiltration

C. Monitor bacterial proliferation

1. Collect 3 leaf discs from inoculated leaves at 1 h post infiltration. The 1 h wait prevents erroneous bacterial numbers from any excess inoculum on the leaf surface. By 1 h post infiltration, the infiltrated leaves should no longer appear wet. Collect leaf discs using 1 cm cork borer from each plant. This is your 0 dpi sample.

2. Homogenize leaf discs in 0.3 ml, 10 mM MgCl₂ in a microcentrifuge tube manually using pellet pestle (Figure 3). For reproducibility and accurate bacterial count homogenize until no leaf pieces are visible. (Figure 4). Increase final volume to 1 ml after complete homogenization.
3. Dilute 10x using 10 mM MgCl₂ and plate 100 µl on King's B medium (one sample per plate) using a glass spreader. Incubate plates at 29 °C till colonies grow (usually 24-48 h). Plate minimum 3 technical replicates per bacterial strain for every genotype. Technical repeats indicate independent leaf extracts from the same set of plants infected with the same bacterial culture.
4. Count the number of bacterial colonies on entire plate, and adjust for dilution factor to determine total bacterial number. Plot bacterial counts as LOG₁₀ values of CFU/unit leaf disc. It is important to adjust the dilution factor to obtain colonies in a countable range (10-300) depending upon the genotype of the plant. It is recommended to repeat the experiment using lower dilutions for plating if colony numbers are less than 10 or using higher dilutions when colony numbers are more than 300 per plate.
5. Repeat steps 1-4 at 3, 4, 6 dpi (or other desired time points). Dilute homogenized tissue 5,000-10,000 times dilution for 3 dpi and later samples. Use minimum 4-5 technical replicates for these time points. Use 3-4 biological replicates (indicates independent infections using independent bacterial culture and set of plants) per bacterial strain and per plant genotype.
6. Hypersensitive reaction related symptoms are visually detectable by 6-7 dpi (Figure 5).



Figure 3. Image of pellet pestle used for homogenization of leaf material



Figure 4. Image of homogenate of infected leaf material

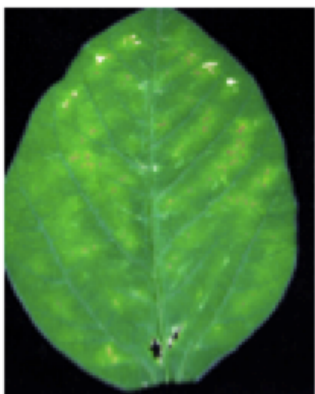


Figure 5. Morphological symptoms associated with the hypersensitive response of *P. syringae* pv. *glycinea* infected soybean leaves

Representative data

Video 1. Airbrush infiltration



1. The protocol has high reproducibility (>90%) in our hands. We normally use at least four biological replicates per genotype for each experiment, and each infection experiment is conducted with a minimum three technical replicates. The standard error for colony count ranges between LOG₁₀ value of 0.01-0.2.
2. See Selote and Kachroo (2010); Selote *et al.* (2013) and (2014); Wang *et al.* (2014) for typical bacterial counts observed on various cultivars.

Notes

1. Care should be taken to avoid damaging the leaves during the inoculation process.
2. It is best to use fully expanded leaves for infection and to inoculate all leaves of one trifoliolate per plant.

3. Spraying the bacterial suspension over the leaf surface is generally sufficient. It is not necessary to saturate leaves with the bacterial suspension as excessive pressure during infiltration can result in wilting of the leaf.

Recipes

1. King's B medium (plates and liquid media)
 - 20 g tryptone
 - 1.5 g K₂HPO₄
 - 10 ml glycerol
 - 15 g agar, make up volume to 1 L with water
 - Autoclave, then add 5 ml of sterile MgSO₄ (1 M)

Acknowledgments

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