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DEPARTMENT OF PLANT AND MICROBIAL BIOLOGY

Dr. Jennifer D. Lewis

October 30, 2019

Dr. Nandita Singh
Senior Science Editor, JoVE

Dear Dr. Singh,

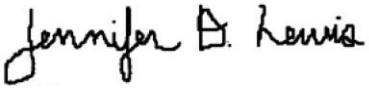
Thank you for offering us the opportunity to submit a revised version of our manuscript (JoVE60805) entitled "High-throughput identification of resistance to *Pseudomonas syringae* pv. *tomato* in tomato using seedling flood assay" from Jana A. Hassan, Ilea J. Chau-Ly and myself.

Please find below our point-by-point reply to all of the comments and concerns of the editor and three expert reviewers. We thank the editor and reviewers for their extensive and insightful comments, and have made revisions according to their suggestions. The modified sections of the manuscript are marked in blue highlight in a separate document (the yellow highlighting for filming was removed in the tracked version).

The manuscript is uploaded as a doc file. The figures are uploaded as psd files. The tables are uploaded as xlsx files.

We hope the updated manuscript fully addresses the editor's and reviewers' concerns and meets the criteria for publication in JoVE.

With best regards,



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TITLE:

High-Throughput Identification of Resistance to *Pseudomonas Syringae* Pv. *Tomato* in Tomato using Seedling Flood Assay

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KEYWORDS:

Pseudomonas syringae, tomato, disease resistance, screen, bacterial growth, phenotypes

SUMMARY:

The seedling flood assay facilitates rapid screening of wild tomato accessions for the resistance to the *Pseudomonas syringae* bacterium. This assay, used in conjunction with the seedling bacterial growth assay, can assist in further characterizing the underlying resistance to the bacterium, and can be used to screen mapping populations to determine the genetic basis of resistance.

ABSTRACT:

Tomato is an agronomically important crop that can be infected by *Pseudomonas syringae*, a Gram-negative bacterium, resulting in bacterial speck disease. The tomato-*P. syringae* pv. *tomato* pathosystem is widely used to dissect the genetic basis of plant innate responses and disease resistance. While disease was successfully managed for many decades through the introduction of the *Pto/Prf* gene cluster from *Solanum pimpinellifolium* into cultivated tomato, race 1 strains of *P. syringae* have evolved to overcome resistance conferred by the *Pto/Prf* gene cluster and occur worldwide.

Wild tomato species are important reservoirs of natural diversity in pathogen recognition, because they evolved in diverse environments with different pathogen pressures. In typical screens for disease resistance in wild tomato, adult plants are used, which can limit the number of plants that can be screened due to their extended growth time and greater growth space requirements. We developed a method to screen 10-day-old tomato seedlings for resistance, which minimizes plant growth time and growth chamber space, allows a rapid turnover of plants, and allows large sample sizes to be tested. Seedling outcomes of survival or death can be treated as discrete phenotypes or on a resistance scale defined by amount of new growth in surviving

seedlings after flooding. This method has been optimized to screen 10-day-old tomato seedlings for resistance to two *P. syringae* strains and can easily be adapted to other *P. syringae* strains.

INTRODUCTION:

Pseudomonas syringae is a Gram-negative pathogenic bacterium that infects a wide range of plant hosts. Bacteria enter the host plant through the stomata or physical wounds and proliferate in the apoplast[1]. Plants have evolved a two-tiered immune response to protect against infection by bacterial pathogens. The first level occurs at the plant cell surface, where pattern recognition receptors on the plant cell membrane perceive highly conserved pathogen-associated molecular patterns (PAMPs) in a process called PAMP-triggered immunity (PTI)[2]. During this process, the host plant upregulates defense response pathways, including deposition of callose to the cell wall, closure of stomata, production of reactive oxygen species, and induction of pathogenesis-related genes.

Bacteria can overcome PTI by utilizing a type III secretion system to deliver proteins, called effectors, directly into the plant cell[3]. Effector proteins commonly target components of PTI and promote pathogen virulence[4]. The second tier of plant immunity occurs within the plant cell upon recognition of the effector proteins. This recognition is dependent on resistance genes, which encode nucleotide-binding site leucine-rich repeat containing receptors (NLRs). NLRs are capable of either recognizing effectors directly or recognizing their activity on a virulence target or decoy[5]. They then trigger a secondary immune response in a process called effector-triggered immunity (ETI), which is often associated with a hypersensitive response (HR), a form of localized cell death at the site of infection[6]. In contrast to gene-for-gene resistance associated with ETI, plants can exhibit quantitative partial resistance, which is dependent on the contribution of multiple genes[7].

P. syringae pv. *tomato* (*Pst*) is the causal agent of bacterial speck on tomato and is a persistent agricultural problem. Predominant strains in the field have typically been *Pst* race 0 strains that express either or both of the type III effectors AvrPto and AvrPtoB. DC3000 (*Pst*DC3000) is a representative race 0 strain and a model pathogen that can cause bacterial speck in tomato. To combat bacterial speck disease, breeders introgressed the *Pto* [*P. syringae pv. tomato*]*/Prf* [*Pto resistance and fenthion sensitivity*] gene cluster from the wild tomato species *Solanum pimpinellifolium* into modern cultivars[8,9]. The *Pto* gene encodes a serine-threonine protein kinase that, together with the *Prf* NLR, confer resistance to *Pst*DC3000 via recognition of the effectors AvrPto and AvrPtoB[10–14]. However, this resistance is ineffective against emerging race 1 strains, allowing for their rapid and aggressive spread in recent years[15,16]. Race 1 strains evade recognition by the *Pto/Prf* cluster, because AvrPto is either lost or mutated in these strains, and AvrPtoB appears to accumulate minimally[15,17,18].

Wild tomato populations are important reservoirs of natural variation for *Pst* resistance and have previously been used to identify potential resistance loci[19–21]. However, current screens for pathogen resistance utilize 4–5-week-old adult plants[20,21]. Therefore, they are limited by growth time, growth chamber space, and relatively small sample sizes. To address the limitations of conventional approaches, we developed a high-throughput tomato *P. syringae* resistance assay using 10-day-old tomato seedlings[22]. This approach offers several advantages over using adult

plants: namely, shorter growth time, reduced space requirements, and higher throughput. Furthermore, we have demonstrated that this approach faithfully recapitulates disease resistance phenotypes observed in adult plants[22].

In the seedling flood assay described in this protocol, tomato seedlings are grown on Petri dishes of sterile Murashige and Skoog (MS) media for 10 days and then are flooded with an inoculum containing the bacteria of interest and a surfactant. Following flooding, seedlings can be quantitatively evaluated for disease resistance via bacterial growth assays. Additionally, seedling survival or death can act as a discrete resistance or disease phenotype 7–14 days after flooding. This approach offers a high-throughput alternative for screening large numbers of wild tomato accessions for resistance to *Pst* race 1 strains, such as *Pst* strain T1 (*Pst*T1), and can easily be adapted to other bacterial strains of interest.

PROTOCOL:

1. Preparation and use of biosafety cabinet

1.1. Wipe down biosafety cabinet with 70% ethanol.

1.2. Close the sash and turn on the ultraviolet light in the biosafety cabinet for 15 min.

1.3. After 15 min, turn off the ultraviolet light in the biosafety cabinet. Lift the sash and turn on the blower for 15 min.

1.4. Wipe all items to be used in the biosafety cabinet with 70% ethanol prior to putting the items into the sterilized cabinet.

1.5. Clean gloves or bare hands with 70% ethanol before working in the biosafety cabinet.

1.6. Work in the center of the biosafety cabinet, away from the blower.

1.7. Use unopened bottles of autoclaved sterile 10 mM $MgCl_2$ and ultrapure H_2O for experiments. Put bottles in the biosafety cabinet and only open them in the sterilized biosafety cabinet, not on the benchtop.

1.8. Use dedicated glass pipettes and pipette tips for work in the sterilized biosafety cabinet. Ensure that these are only opened in the biosafety cabinet, never on the benchtop.

1.9. After use of the biosafety cabinet, autoclave all waste (except bleach waste) and wipe down the surface with 70% ethanol.

2. Preparation of plant media

2.1. Weigh out and dissolve 0.5x MS basal salts in ultrapure H_2O. Weigh out 0.8% bacto agar and then add to dissolved 0.5x MS.

2.2. Autoclave and allow the media to cool in 50 ˚C water bath for 1 h prior to pouring or pipetting.

2.3. To ensure that plates are not overfilled, mark polystyrene disposable sterile 100 x 25 mm plates to a fill level of 40 mL. Pour media into 100 x 25 mm sterile plates in a sterilized biosafety cabinet.

3. Preparation of plant materials and growth conditions

3.1. Place tomato seeds in a 2.2 mL microcentrifuge tube and add 2.0 mL of 50% bleach solution.

3.2. Rock the tube on a rocker for 25 min.

3.3. After 25 min, remove the seeds from the rocker and remove the bleach solution with a pipette in the sterile biosafety cabinet. Ensure that all the bleach is removed.

3.4. Add 2 mL of sterile ultrapure H_2O to wash the seeds. Invert the tube 5x.

3.5. Remove the liquid from the tube with a pipette.

3.6. Repeat steps 3.3–3.5 to wash the seeds 4x more.

3.7. Add 2 mL of sterile ultrapure H_2O and pour the seeds into an empty sterile Petri dish.

3.8. Flame forceps in ethanol and allow to cool prior to transferring and evenly spacing seeds on 100 x 25 mm plates containing 0.5x MS + 0.8% agar media.

3.9. Transfer 5–7 seeds in a line across the middle of one plate and seal the edges of the plates with surgical tape (1.25 cm x 9.1 m).

3.10. Stratify the sterilized seeds at 4 ˚C in the dark for at least 3 days to synchronize germination. Ensure that the plates are stacked flat and face up, so that the seeds do not shift on the plate.

3.11. Vertically orient the plates so that the roots will grow down along the surface of the plate, with the line of seeds oriented horizontally, when transferring to the growth chamber.

NOTE: Set the growth chamber to 22 ˚C and provide 16 h of light at a light intensity of ~200–220 µE $meter^{-2}$ s^{-1} and 8 h of darkness.

3.12. Prior to flooding, grow seedlings for 10 days in the growth chamber at which point seedlings typically display fully emerged and expanded cotyledons and emerging first true leaves (**Figure 1**).

[place figure 1 here]

4. Preparation of King's B[23] (KB) media

4.1. Fill beaker with 500 mL of ultrapure H_2O and stir on a stir plate.

4.2. Completely dissolve 20 g of bacto peptone, 1.5 g of anhydrous K_2HPO_4, and 12.5 mL of glycerol in a beaker with ultrapure H_2O.

4.3. Pour the dissolved mixture into a 1 L graduated cylinder and bring up to a 1 L final volume with ultrapure H_2O.

4.4. Pour the broth back into the beaker and stir until mixed.

4.5. Weigh out 7.5 g of bacto agar into two 500 mL glass bottles and add 500 mL of KB broth from step 4.4 into each bottle. Autoclave for 20 min.

4.6. Remove the bottles from the autoclave and swirl gently to distribute the agar.

4.7. Transfer the bottles to a 50 ˚C water bath for 1 h.

4.8. After 1 h, transfer the bottle to the biosafety cabinet and under aseptic conditions, add 1,600 µL of sterile 1 M $MgSO_4$, and appropriate antibiotics to the media.

NOTE: For rifampicin resistant strains *Pst*DC3000 and *Pst*T1, use rifampicin dissolved in dimethylformamide at a final concentration of 50 µg/mL. Use cycloheximide dissolved in ethanol at a final concentration of 50 µg/mL to prevent fungal growth on the plates.

4.9. Swirl the media gently to mix and then pour to cover the bottom of the plates.

4.10. Allow at least 1 h for the plates to solidify before storing them upside down at 4 ˚C.

5. Maintenance of bacterial strains and culture conditions

5.1. Maintain a glycerol stock from single colony of bacteria as 1 mL of saturated bacterial culture and 333 µL of sterile 80% glycerol at -80 ˚C.

5.2. Patch bacteria (i.e., *Pst*T1) from a glycerol stock onto KB agar with appropriate antibiotics (section 4).

5.3. Allow the bacteria to recover for 2 days at 28 ˚C before streaking fresh bacteria onto selective KB agar using a flat, sterile toothpick.

5.4. Streak fresh bacteria from the glycerol stock onto appropriate selective KB agar using a flat, sterile toothpick.

NOTE: Ensure that the patched glycerol stock is not more than 2 weeks old.

5.5. For *Pst*DC3000, incubate the KB plate at 28 ˚C for 24 h prior to using bacteria in the flood experiment.

5.6. For *Pst*T1, incubate the KB plate at 28 ˚C for 48 h prior to using bacteria in the flood experiment.

6. Preparation of *Pst*T1 inoculum

6.1. Aseptically resuspend the bacteria in sterile 10 mM $MgCl_2$ to an optical density at 600 nm (OD_{600}) of 0.1, or approximately 5 x 10^7 colony forming units (CFU)/mL).

6.2. Perform serial dilutions using sterile 10 mM $MgCl_2$ solution in the biosafety cabinet. For *Pst*T1, use a spectrophotometer to make inoculum with a starting concentration of OD_{600} = 0.1.

6.3. For *Pst*T1, make a 1/10 dilution from the initial resuspension at OD_{600} = 0.1 to obtain a serial dilution at a concentration of OD_{600} = 0.01.

6.4. Using the serial dilution at OD_{600} = 0.01 from step 6.3, make a 3/4 dilution to obtain a final OD_{600} = 0.0075.

6.5. Make a 1/10 dilution of non-ionic organosilicone surfactant copolymer $C_{13}H_{34}O_4Si_3$ (i.e., surfactant) in 10 mM $MgCl_2$ and vortex for 15 s. Add the 1/10 stock of surfactant to the last serial dilution (OD_{600} = 0.0075) to a final concentration of 0.015% and swirl well to mix.

7. Preparation of *Pst*DC3000 inoculum

7.1. Aseptically resuspend bacteria in sterile 10 mM $MgCl_2$ to an optical density at 600 nm (OD_{600}) of 0.1 (approximately 5 x 10^7 CFU/mL).

7.2. Perform serial dilutions using sterile 10 mM $MgCl_2$ solution in the biosafety cabinet. For *Pst*DC3000, use a spectrophotometer to make inoculum with a starting concentration of OD_{600} = 0.1.

7.3. For *Pst*DC3000, make a 1/10 dilution from the initial resuspension at OD_{600} = 0.1 to obtain a serial dilution at a concentration of OD_{600} = 0.01.

7.4. Using the serial dilution at OD_{600} = 0.01 from step 3, make a 1/2 dilution to obtain a final OD_{600} = 0.005.

7.5. Make a 1/10 dilution of surfactant in 10 mM $MgCl_2$ and vortex for 15 s. Add the 1/10 stock of surfactant to the last serial dilution (OD_{600} = 0.005) to a final concentration of 0.015% and swirl well to mix.

8. Tomato seedling flood method

8.1. Take the plates with the 10-day-old seedlings out of the growth chamber and put in the biosafety cabinet to prepare the plates for flooding.

8.2. Remove the surgical tape from two plates.

8.3. Set a timer for 3 min. Measure 6 mL of final inoculum (*Pst*T1 OD_{600} = 0.0075 [section 6] or *Pst*DC3000 OD_{600} = 0.005 [section 7]) and transfer 6 mL of inoculum to each plate with the 10-day-old seedlings.

8.4. Gently push the seedlings down into the inoculum with a sterile pipette tip. Start the timer.

8.5. Hold one plate in each hand. Tilt the front of the plate down to accumulate inoculum and mainly submerge the cotyledons and leaves of the seedlings.

8.6. Swish side to side 5–7x and then tip the plates back to cover the roots and the whole plate.

8.7. Tilt the plates down again to submerge the cotyledons and leaves, and repeat for a total of 3 min.

8.8. Pour the inoculum off the plates, set the plates down on a flat surface and then pour off any residual inoculum a second time.

8.9. Rewrap the plates with surgical tape and repeat steps 8.2–8.8 for any remaining plates.

8.10. Re-incubate the plates in the growth chamber (see step 3.11 NOTE) after all plates have been flooded.

8.11. Phenotype after 7–10 days for *Pst*DC3000 or 10–14 days for *Pst*T1 (section 11). If carrying out bacterial growth assays, collect leaf tissue after 4 days (sections 9 and 10) and then phenotype (section 11). Alternatively, perform phenotypic analysis and bacterial growth assays on separate sets of plants.

9. Surface sterilization of cotyledons for bacterial growth assay

9.1. Four days after flooding and re-incubating the seedlings in the growth chamber (section 8), remove the plates with the tomato seedlings from the growth chamber.

9.2. Number the individual seedlings on the bottom exterior of the plate where the seedling attaches to the plate for each genotype.

9.3. Label sterile 1.5 mL microcentrifuge tubes with the individual seedling numbers and use clean forceps to drop one 3 mm sterile borosilicate bead into each tube for use with a bead beater. (See NOTE in step 10.1.)

9.4. Pipette 200 µL of 10 mM $MgCl_2$ into each tube and close tubes.

9.5. Prepare 70% ethanol and pour 100 mL into a clean beaker. Pour 100 mL of sterile ultrapure H_2O into a separate, clean beaker.

9.6. Clean stainless steel straight fine-point forceps with serrated tips with ethanol. Open the plate slightly to allow aseptic removal of one cotyledon with the clean forceps.

9.7. Pinch the petiole at the base of the cotyledon to remove a leaf and drop into the beaker with 70% ethanol to surface-sterilize for 10 s. Rinse the cotyledon in ultrapure H_2O for 10 s.

9.8. Place the cotyledon on a paper towel and blot dry with delicate science wipes.

9.9. Individually weigh each cotyledon after surface sterilization and blotting, and record the weight.

9.10. Place the cotyledon in a previously prepared 1.5 mL microcentrifuge tube (from steps 9.3 and 9.4) labelled with the corresponding genotype and individual number.

9.11. Reseal the plates with sterile tape and re-incubate the seedlings in the growth chamber (see step 3.11 NOTE).

10. Bacterial growth assay

10.1. Using samples from step 9.10, homogenize the tissue using the bead beater in 10 mM $MgCl_2$ for 1–2 min. If the tissue is not adequately macerated, homogenize again.

NOTE: Many manufacturers produce bead beater homogenizers. The number and type of beads, as well as the homogenization time and speed (if programmable) should be optimized for each type of homogenizer. Ensure that the samples do not overheat during homogenization.

10.2. Add 800 µL of 10 mM $MgCl_2$ to each tube containing macerated tissue from step 10.1 and invert several times to mix.

10.3. Prepare serial dilutions for each sample in 10 mM $MgCl_2$ in 96 well plate (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) using a multichannel pipette (**Figure 2A**).

10.4. Pipette 5 µL from each dilution series using a multichannel pipette onto a KB agar plate (150 mm x 15 mm) with cycloheximide and appropriate selection for the bacterial strain of interest (see step 4.8 NOTE). Let the plates dry completely.

10.5. Incubate the plate upside down at 28 ˚C for 36 h, then visualize (**Figure 2B**) the colonies on the plates using a dissecting microscope to determine if the colonies are large enough to count.

NOTE: If the colonies are not large enough, re-incubate the plates and recheck the size of the colonies every few hours. Typically, the colonies are countable ~36–48 h after incubation.

[place figure 2 here]

10.6. Count the colonies under a dissecting microscope before they merge (**Figure 2B**). Count the colonies from the dilution series plates with fewer than 100 colonies.

10.7. After obtaining colony counts (**Figure 2B**), normalize the counts to 0.01 g of tissue for seedlings and convert to log bacterial growth (**Table 1**).

NOTE: The average mass of one Moneymaker-*PtoS* cotyledon is 0.01 g and is empirically determined[22].

[place Table 1 here]

10.8. For wild accessions and other lines with complex genetic backgrounds, correlate the level of bacterial growth in individual seedlings with their phenotype as described in section 11.

11. Phenotyping for resistance

11.1. Remove the plates from the growth chamber and phenotype individual seedlings for death (due to disease) or survival (due to resistance) after 7–14 days.

11.2. Phenotype plants infected with a highly virulent strain such as *Pst*DC3000 earlier, at 7–10 days after flood inoculation.

11.3. Phenotype plants infected with *Pst*T1 at 10–14 days after flood inoculation.

11.4. Determine a scoring system based on the range of resistance phenotypes observed. Record binary phenotypes for cultivars, isogenic lines, and wild accessions with consistent, strong to intermediate resistance phenotypes (**Figure 4A**, **4B**).

11.5. If the seedling displays new growth from the apical meristem within the time frame for phenotyping count it as a survival. If the seedling has a brown apical meristem and displays no new, green vegetative growth, count it as a death.

[place figure 3 here]

11.6. Record phenotypes on a disease spectrum for populations, such as F2 mapping populations, with a wide range of resistance phenotypes (**Figure 4C**).

11.7. Carefully monitor the seedlings for the appearance of disease symptoms and death to identify the appropriate window for phenotyping.

[place figure 4 here]

REPRESENTATIVE RESULTS:

Detection of *PtoR*-mediated immunity in cultivars and isogenic lines using the seedling resistance assay

Figure 5 shows representative results for Moneymaker-*PtoR* and Moneymaker-*PtoS* cultivars 7–10 days after flooding with *Pst*DC3000. Prior to infection, 10-day-old seedlings displayed fully emerged and expanded cotyledons and emerging first true leaves. The seedlings were flooded with 10 mM $MgCl_2$ + 0.015% surfactant as a negative control (data not shown) and *Pst*DC3000 at an optical density of 0.005 + 0.015% surfactant. The seedlings were phenotyped 7–10 days after flooding (**Figure 5**). Individual seedlings from genotypically homogenous lines, such as Moneymaker-*PtoR* and Moneymaker-*PtoS* give highly consistent and binary phenotypes in the seedling flood assay. When Moneymaker-*PtoR*, which carries the *Pto/Prf* gene cluster (n = 5), was treated with *Pst*DC3000 at the optimal concentration of OD_{600} = 0.005, resistance due to *PtoR*-mediated immunity was strong and was typified by new, green vegetative growth in all individuals[22]. Near-isogenic Moneymaker-*PtoS* seedlings (n = 5), which cannot recognize the *Pst*DC3000 effectors AvrPto or AvrPtoB, died quickly within 7 days after flooding and characteristically had brown apical meristems, bacterial speck, chlorosis, and no signs of new, green vegetative growth (**Figure 5**).

[place figure 5 here]

Phenotypic screening of wild accessions using the seedling resistance assay

Figure 6 shows representative results for seedlings of susceptible and resistant accessions 10–14 days after flooding with *Pst*T1. Susceptible accessions include RG-*PtoR*, *S. pimpinellifolium* LA1375, and *S. pimpinellifolium* LA1606, and resistant accessions include *S. neorickii* LA1329. Ten-day-old seedlings were flooded with 10 mM $MgCl_2$ + 0.015% surfactant as a negative control, and *Pst*T1 at an optical density of 0.0075 + 0.015% surfactant. The seedlings were phenotyped at least 10 days after flooding, as *Pst*T1-infected seedlings died more slowly than *Pst*DC3000-infected seedlings. Mock-inoculated seedlings were green, healthy, and actively growing. This control is important to ensure that the accessions are not sensitive to the concentration of surfactant, and to ensure there is no bacterial contamination. Susceptible accessions (Rio Grande-*PtoR* [n = 7], *S. pimpinellifolium* LA1375 [n = 7], and *S. pimpinellifolium* LA1606 [n = 5]) were dead, had brown apical meristems, and lacked new growth 10–14 days after inoculation with *Pst*T1. In contrast, two *S. neorickii* LA1329 (n = 3) seedlings displayed a high level of new, green growth and survived infection with *Pst*T1 (**Figure 6**). Three LA1329 seedlings did not germinate. Typically, 5–7

individuals were screened for each accession in a primary screen to determine the prevalence of resistance in the population. When a more genetically complex wild accession, such as LA1329, is flooded with *Pst*T1, the resistance phenotypes display slightly more variability among individual seedlings, compared to Moneymaker-*PtoR* treated with *Pst*DC3000. However, the resistance phenotypes were usually less variable than those seen in F2 mapping populations. Thus, binary phenotyping criteria was used for LA1329.

[place figure 6 here]

Quantitative assessment of bacterial growth using the seedling flood assay

To confirm that the observed resistance in LA1329 to *Pst*T1 resulted in lower bacterial growth, bacterial growth assays were carried out in tomato seedlings. The level of *Pst*T1 growth in Moneymaker-*PtoS* and *S. neorickii* LA1329 was determined 4 days post-infection. Moneymaker-*PtoS* is a near-isogenic line with consistent susceptibility among individual seedlings. Wild accessions such as *S. neorickii* LA1329 are often more genetically complex. LA1329 displays approximately 60% resistance to *Pst*T1 across the population[22]. Because seedlings may drop their cotyledons after infection, one seedling was grown on each plate to correlate bacterial growth in the harvested cotyledon with overall seedling survival or death as determined phenotypically at least 10 days after flooding. The bacterial counts on day 4 for each seedling were normalized to 0.01 g of tissue and converted to log growth (CFU/0.01 g($\log_{10}$)). Log growth for phenotypically resistant LA1329 seedlings ($LA1329^{RES}$) or phenotypically susceptible seedlings ($LA1329^{SUS}$) were separately pooled and compared to each other and the susceptible cultivar Moneymaker-*PtoS*. For example, there was a 1.7 log difference in bacterial growth between $LA1329^{RES}$ (log 6.3) and $LA1329^{SUS}$ (log 8.0), and a 1.6 log difference between $LA1329^{RES}$ (log 6.3) and Moneymaker-*PtoS* (log 7.9) (**Figure 7**). Therefore, phenotypic resistance correlated with quantitative resistance in the seedling assays.

[place figure 7 here]

FIGURE LEGENDS:

Figure 1: Developmental stage of typical 10-day-old tomato seedlings. Rio Grande-*PtoR* tomato seeds were sterilized, plated, and stratified for at least 3 days in the dark at 4 ˚C. The seedlings were grown on 0.5x MS plates for 10 days at 22 ˚C before being flooded. Typically, at 10 days the cotyledons are fully expanded, and the first true leaves are beginning to emerge.

Figure 2: Serial dilutions for seedling bacterial growth assays. (**A**) Macerated leaf tissue from infected plants is diluted prior to colony counting. Dilutions are performed in a 96 well plate (10^0 is undiluted). Typically, dilutions are made from 10^{-1} to 10^{-5}. (**B**) Plating dilutions for bacterial colony counts. A total of 5 µL of each column of the dilution series is plated, from most dilute to most concentrated. After the colonies have fully dried, the plate is incubated at 28 ˚C for 36–48 h. Colonies are counted under a 10x dissecting microscope.

Figure 3: Schematic representation of a tomato seedling. Different parts of a tomato seedling are depicted, including the hypocotyl, cotyledons, epicotyl, shoot apical meristem, and true leaves.

Figure 4: Schematic representation of expected phenotypes for seedling resistance and death in various genetic backgrounds. (**A**) Seedlings of Rio Grande-*PtoR* and the near-isogenic cultivar Rio Grande-*PtoS* are displayed 7 days after flooding with *Pst*DC3000 (OD_{600} = 0.005) + 0.015% surfactant. Rio Grande-*PtoR* displays consistent resistance, and Rio Grande-*PtoS* displays consistent susceptibility to infection with *Pst*DC3000. These lines give rise to discrete and binary phenotypes. (**B**) Seedlings of a wild accession, such as *Solanum neorickii* LA1329, are shown 10 days after flooding with *Pst*T1 (OD_{600} = 0.0075) + 0.015% surfactant. Seedlings display phenotypic variability but were recorded as binary phenotypes. The amount of phenotypic variability and the method of phenotyping (binary resistance or resistance spectrum) will depend on the particular accession tested. (**C**) Mapping populations generated by outcrossing wild accessions to susceptible cultivars may display a wider spectrum of phenotypes in F2 segregating populations. In this case, it may be most appropriate to record seedling phenotypes on a spectrum. Highly susceptible seedlings from a mapping population may be phenotyped for death as early as day 7 when flooded with *Pst*T1, and typically show a brown apical meristem, no to very little extension of the epicotyl, and no new, green vegetative growth. The apical meristem of susceptible seedlings may stay green or very light brown for more time, and there may be some extension of the epicotyl and very little vegetative growth, which turns brown and arrests by day 10. Individual seedlings can be phenotyped for resistance based on the amount of new and ongoing vegetative growth by day 14. Seedlings can then be grouped based on the phenotypes described above into different categories of resistance such as weak, medium, or strong resistance.

Figure 5. Phenotypic characterization of resistance or disease symptoms 7–10 days post-infection in a cultivar. Moneymaker-*PtoR* and Moneymaker-*PtoS* tomato seedlings were grown on 0.5x MS plates for 10 days before being flooded with *P. syringae* pv. *Tomato* DC3000 (OD_{600} = 0.005) + 0.015% surfactant. Moneymaker-*PtoR* seedlings survived (n = 5) and Moneymaker-*PtoS* seedlings (n = 5) died. The number of surviving seedlings for each genotype out of the total number tested is shown. Scale bar = 1 cm.

Figure 6. Phenotypic characterization of resistance or disease symptoms 10–14 days post-infection in wild accessions. Rio Grande-*PtoR*, *S. pimpinellifolium* LA1606, *S. pimpinellifolium* LA1375 and *S. neorickii* LA1329 tomato seedlings were grown on 0.5x MS plates for 10 days, and then flooded with *Pst*T1 (OD_{600} = 0.0075) + 0.015% surfactant. The number of surviving seedlings for each wild accession out of the total number tested is shown. Scale bar = 1 cm.

Figure 7. Resistant *Solanum neorickii* LA1329 seedlings support lower bacterial growth than Moneymaker-*PtoS* or susceptible *S. neorickii* LA1329. Bacterial counts were determined 4 days post-inoculation from *S. neorickii* LA1329 (n = 14) and Moneymaker-*PtoS* (n = 10) seedlings infected with *Pst*T1 and normalization was performed to 0.01 g of tissue. For LA1329, the two phenotypic groups, susceptible (SUS) or resistant (RES), were observed and counted separately. Above the bar * = statistically significant difference determined by a one-factor analysis of

variance. A general linear model procedure ($p < 0.001$) followed by a multiple comparison of means using Tukey's post hoc test was used. Error bars = standard error. The figure indicates one representative experiment.

Table 1. Sample calculations for seedling bacterial growth assay. Sample calculations demonstrate how to normalize bacterial counts and determine log bacterial growth.

DISCUSSION:

A protocol for flood inoculation with *Pst*DC3000 or *Pst*T1 optimized to detect resistance to these bacterial strains in tomato seedlings is described. There are several critical parameters for optimal results in the seedling resistance assay, including bacterial concentration and surfactant concentration, which were empirically determined[22]. For *Pst*DC3000, the optical density was optimized to achieve complete survival on a resistant cultivar containing the *Pto/Prf* cluster and complete death on a susceptible cultivar lacking the *Pto/Prf* cluster[22]. For a strain such as *Pst*T1, where there are no known resistant varieties, the optical density was optimized to be the lowest possible for consistent and complete plant death[22]. Uppalapati et al.[24] designed a tomato seedling assay to investigate the pathogenesis of *Pst*DC3000 and the virulence function of coronatine. In this virulence assay, infections were performed using bacteria concentrated to an OD_{600} of 0.1[24], 20x higher than the optical density of strains used in our resistance assay. Recognition of *Pst*DC3000 effectors AvrPto and AvrPtoB in tomato seedlings carrying the *Pto/Prf* gene cluster results in ETI and a macroscopic HR[22]. In the context of a strong immune response such as ETI, a lower bacterial titer was used for *Pst*DC3000 to avoid overwhelming genetic resistance from the *Pto/Prf* gene cluster[22]. In addition, these results suggest that a high bacterial concentration could overwhelm weaker immune responses such as PTI or quantitative partial resistance, where multiple genes contribute to the overall phenotype. Surfactant is necessary for the bacteria to adhere to the leaf surface; however, high concentrations can cause chlorosis of the leaf[22]. We previously tested a range of surfactant concentrations to empirically determine the ideal concentration in 10-day-old tomato seedlings[22]. When testing new species that may differ in their sensitivity to surfactant, the surfactant concentration should be optimized to identify a concentration that does not cause damage or chlorosis in the absence of bacteria. Appropriate assay conditions will require optimization of a surfactant concentration that does not cause damage, and a bacterial concentration that causes disease in all susceptible controls.

Additional critical parameters for success in the seedling flood assay include using seedlings at specific developmental stages (10-day-old seedlings) (**Figure 1**), maintaining stable growth chamber conditions (light intensity of about 200 µE m^{-2} s^{-1}, constant temperature of 22 ˚C, 16 h of light) and performing experiments in a sterile biosafety cabinet. Media volume above 45 mL or below 35 mL may affect consistent death of susceptible controls, because the volume may impact the surrounding microenvironment of the seedlings on the plate. For example, differences in relative humidity inside the sealed plates could affect the infectivity of the bacteria and the ability of the plants to survive infection. Sterile technique is critical, because contamination on the plates may confound the source of death or susceptibility in seedlings. In addition, because plant-pathogen interactions are affected by the circadian clock[24–26], it is recommended that the plants be infected at a consistent time of the day.

Pst is a foliar pathogen that preferentially colonizes the aerial parts of tomato seedlings, including the cotyledons[24] (**Figure 3**). Therefore, qualitative phenotyping in the seedling flood assay focuses on growth and disease symptoms in aerial portions of the seedling, and tissue for the bacterial growth assay is sampled from the cotyledons for quantitative analysis. After flood inoculation, seedlings may die within 7–10 days after inoculation with *Pst*DC3000 or 10–14 days after inoculation with *Pst*T1, as discussed in section 11. Seedling death is visualized by a brown apical meristem, arrested epicotyl elongation, and/or arrested vegetative growth. If different bacterial strains are used, the timing will have to be empirically determined. In addition, the progression of disease on control plants should be monitored daily after flooding until a consistent time frame from the onset of disease symptoms to seedling death can be identified. Depending on the genotypes and treatments used in the flood assay, seedling phenotypes can be recorded as binary phenotypes or on a disease spectrum (**Figure 4**). A broader spectrum of phenotypes may be observed when flood inoculating F2 mapping populations from wild tomato accessions crossed to susceptible cultivars (**Figure 4C**). It may be best to phenotype segregating populations on a disease spectrum depending on how quickly the seedling dies and the degree of new vegetative growth and branching (**Figure 4C**). The seedling flood assay can also be used in conjunction with the seedling bacterial growth assay to quantitatively assess levels of bacterial growth associated with qualitative phenotypes in individual seedlings (**Figure 7**). Very large reductions (i.e., ~log 3) in bacterial growth or strong resistance in resistant seedlings of a wild accession compared to a susceptible cultivar suggest that the underlying genetic basis of resistance may be due to ETI[22]. Smaller reductions in bacterial growth (i.e., ~log 1.7), as observed in LA1329 seedlings, may be due to the contribution of weaker resistance from quantitative trait loci and/or PTI. Thus, the seedling growth assay can be an important tool in further characterizing resistance in wild tomato lines.

Typically, genetic screens have been performed on four- to five-week-old adult tomato plants to identify the genetic basis of *P. syringae* resistance in wild accessions[20,21]. Adult tomato plants require much longer growth times, require more space in the growth chamber, and are much larger plants, which means that usually few individuals are screened for each line. The seedling flood assay provides a powerful, alternative approach in the identification of *P. syringae* resistance in wild tomato accessions. Screening at the seedling stage permits a large sample size to be tested which can be particularly advantageous in detecting resistance in genetically complex populations. Reduced growth chamber space requirements and growth time facilitate a high-throughput approach and rapid detection of natural resistance in wild accessions to emerging pathogens. Furthermore, *P. syringae* resistance that was identified at the seedling stage in this assay is not restricted to the developmental stage. *S. neorickii* LA1329 and *S. habrochaites* LA1253 were initially identified at the seedling stage and also display resistance to *Pst*T1 in adult plants as previously described[22].

The seedling flood assay is a versatile protocol that can be modified and optimized to detect host resistance to other *P. syringae* strains. It could potentially be further applied in the context of different bacterial pathogens of tomato, such as the *Xanthomonas* species. This method will expedite the search for new sources of disease resistance to bacterial pathogens.

ACKNOWLEDGMENTS:

We thank Jamie Calma for testing the effect of media volume on disease or resistance outcomes. We thank Dr. Maël Baudin and Dr. Karl J. Scheiber from the Lewis Lab for providing constructive comments and suggestions on the manuscript. Research on plant immunity in the Lewis laboratory was supported by the USDA ARS 2030-21000-046-00D and 2030-21000-050-00D (JDL), and the NSF Directorate for Biological Sciences IOS-1557661 (JDL).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

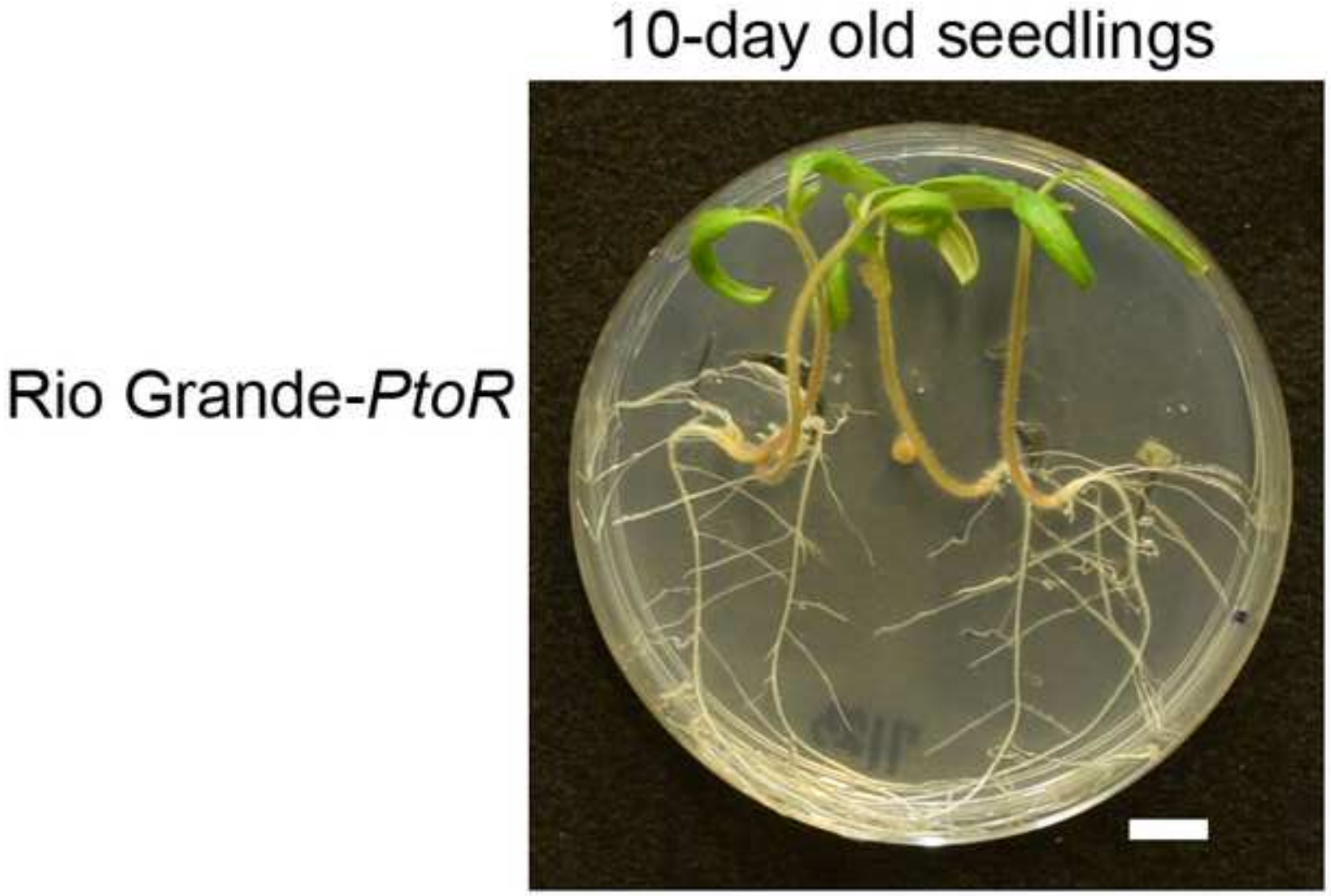


Figure 2

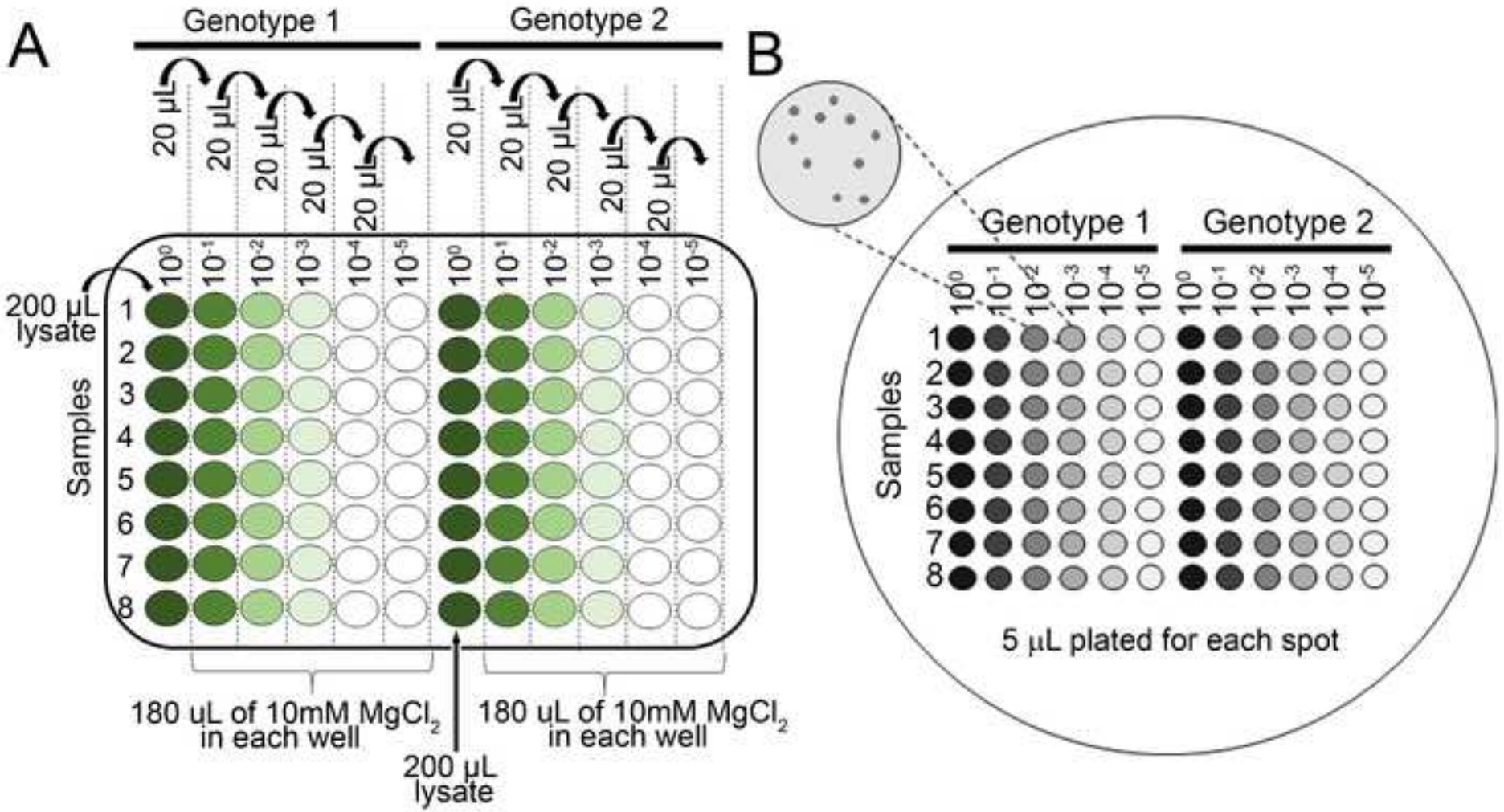
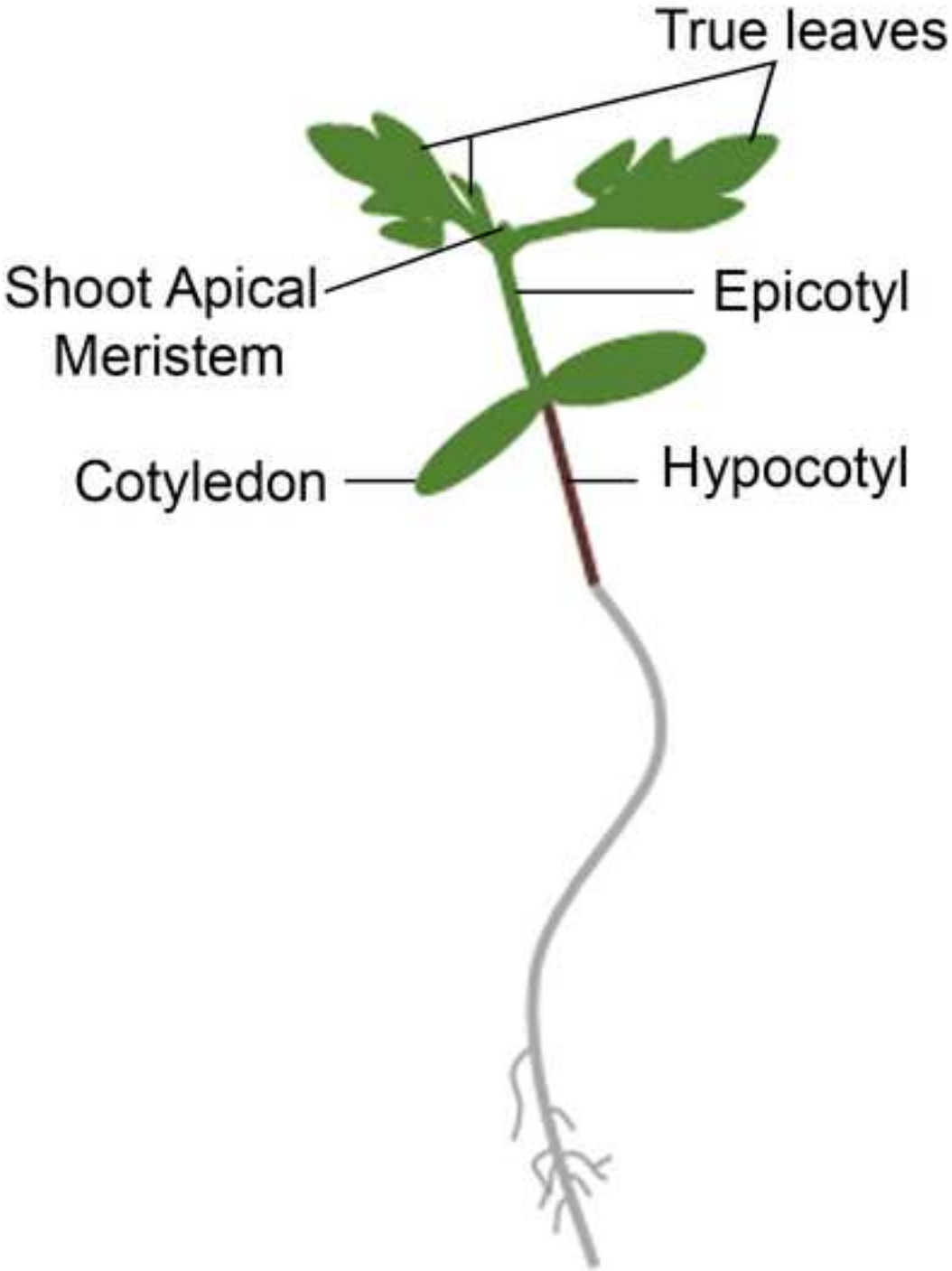


Figure 3



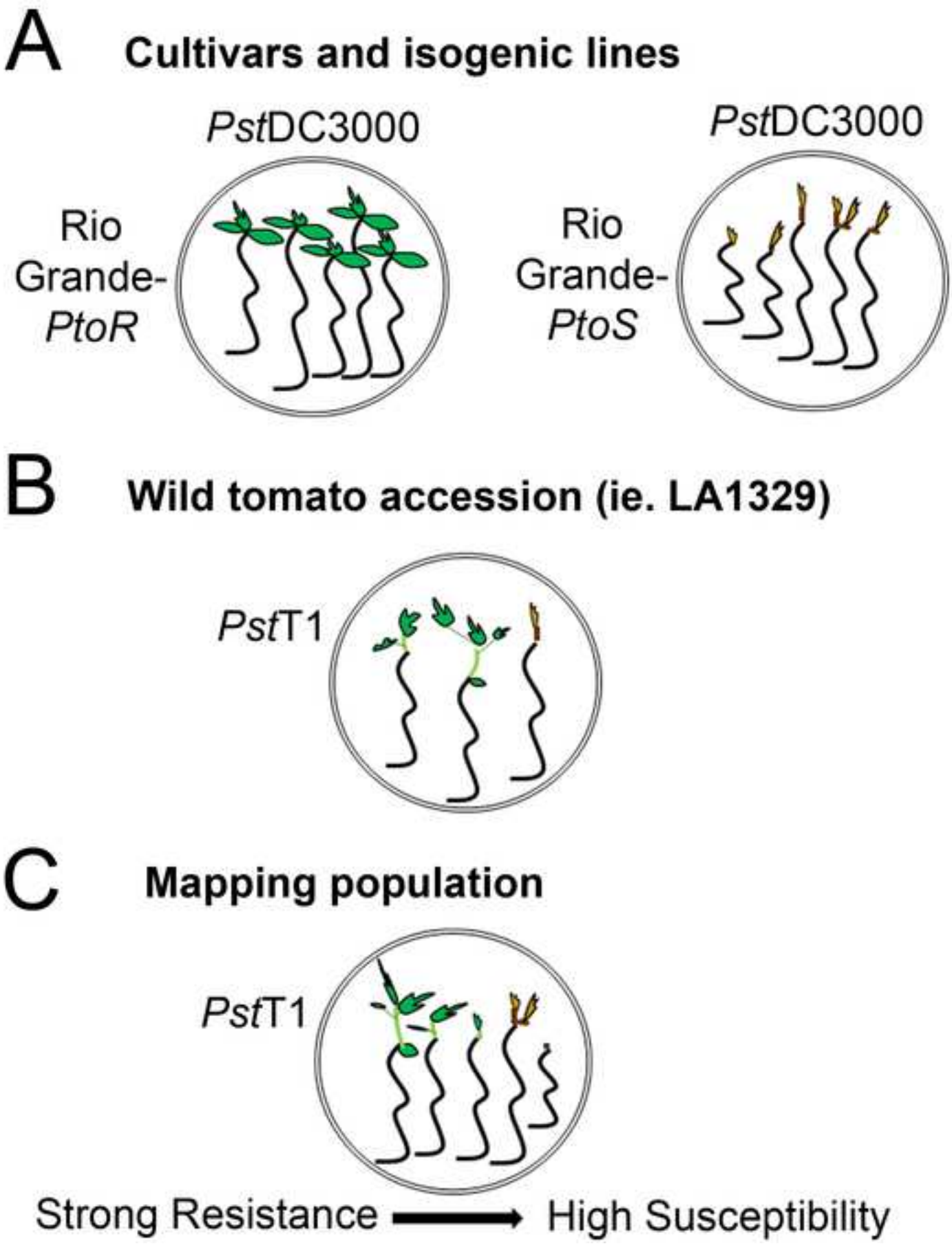


Figure 5

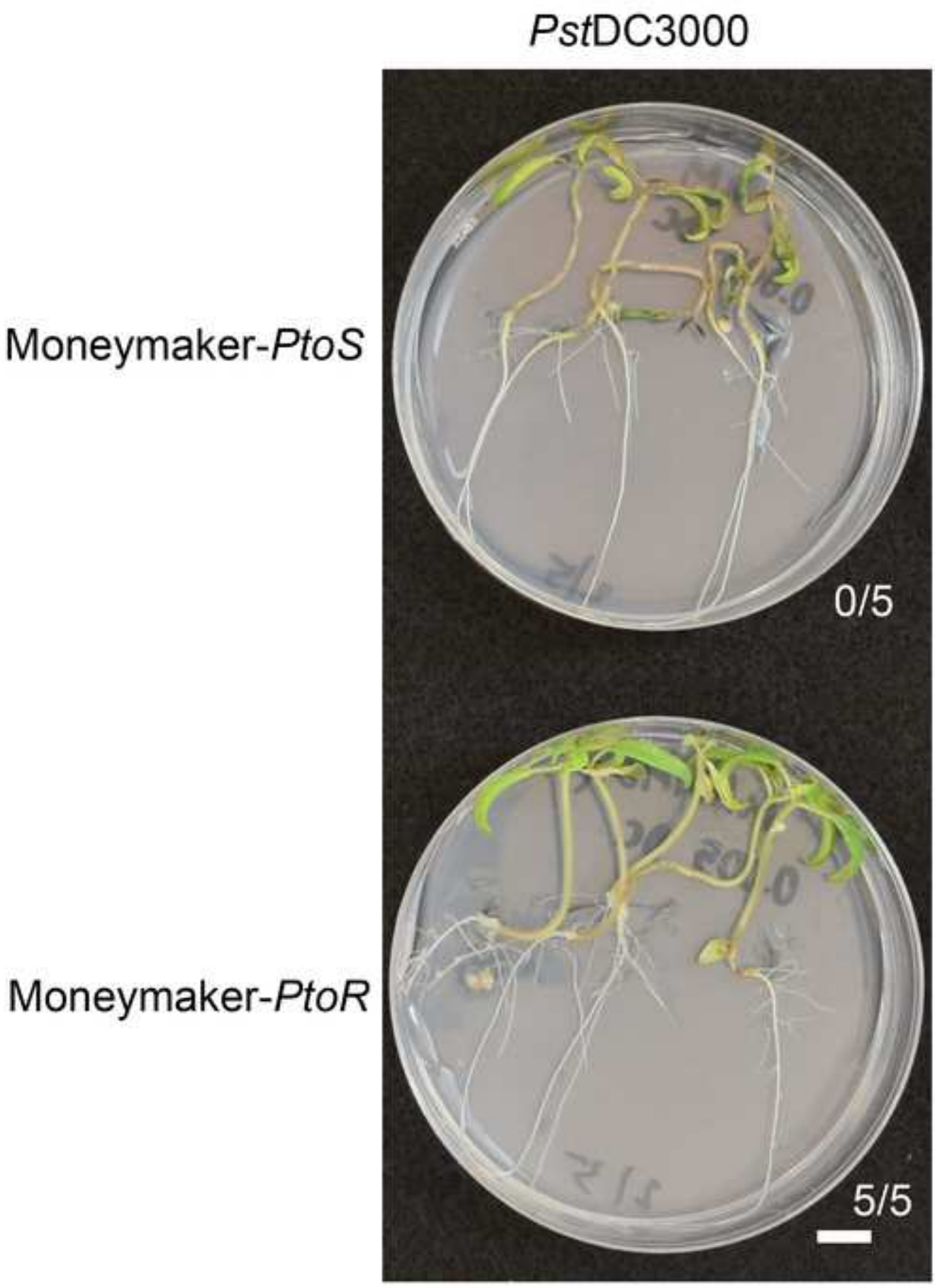


Figure 6

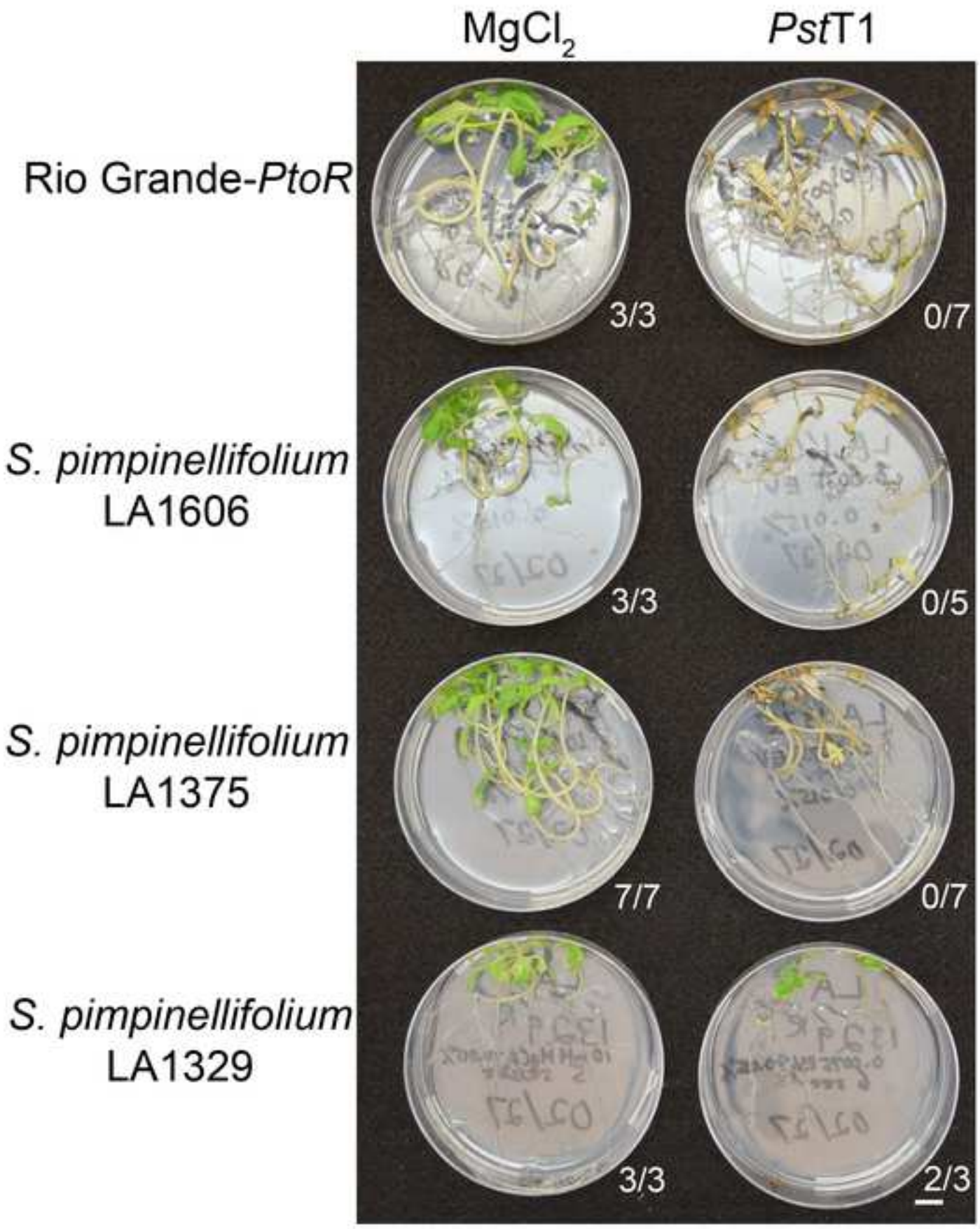


Figure 7

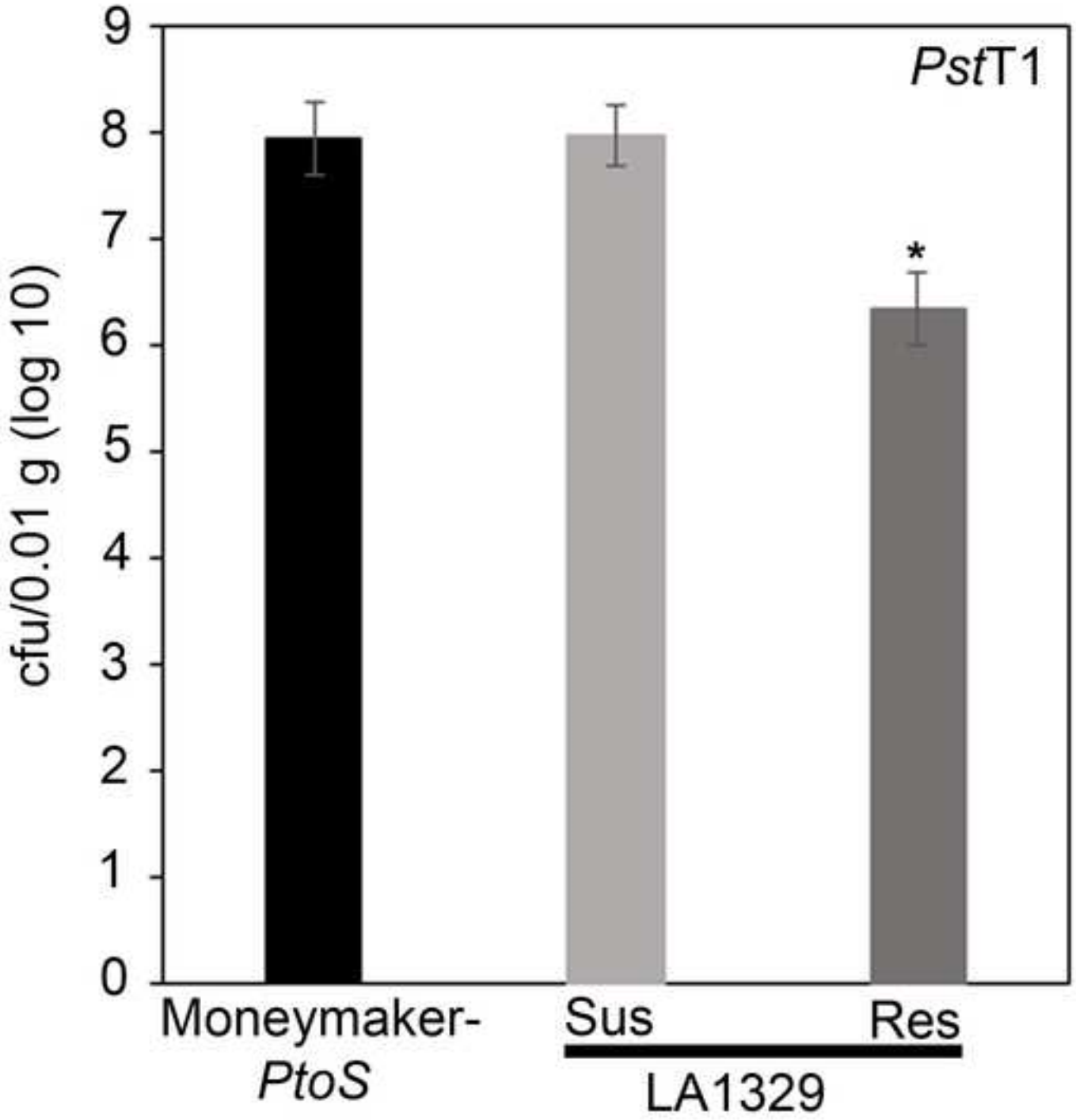


Table 1

Genotype[1] Column A	**Tissue Weight (g) Column B**	**# of colonies in a spot Column C**	**Dilution factor for spot[2] Column D**	**Adjusted # of Colonies[3] Column E**	**Dilution factor for serial dilution Column F**	**Total # of Colonies Column G (cfu/0.01 g)[4]**	**Average # of Colonies (cfu/0.01 g) Column H**
Sample 1	0.004 g	10	200	calculated as: (C2 x 0.01 g) / B2 = 25	1000	calculated as: (D2 x E2 x F2) = 5000000	average for sample 1 through last sample: (ie. average G1:G3) = 7000000
Sample 2	0.003 g	15	200	50	1000	10000000	
Sample 3	0.002 g	6	200	30	1000	6000000	

[1]Data shown for 3 samples

[2]Based on plating 5 μL x 200 for 1 mL

[3]Cotyledons are too small to core so colony counts were normalized to 0.01 g of tissue based on the average mass of one MoneyMaker-*Ptc*

[4]Adjusted per mL based on volume plated

Average Log Growth (cfu/0.01 g (log_{10})) Column I

log of average
ie. log(H2)=6.85

S cotyledon (data not shown)

Name of Material/ Equipment	Company	Catalog Number
3M Tape Micropore 1/2" x 10 YD CS 240 (1.25 cm x 9.1 m)	VWR International	56222-182
3mm borosilicate glass beads	Friedrich&Dimmock	GB3000B
Bacto peptone	BD	211677
Bacto agar	BD	214010
Biophotometer Plus	Eppendorf	E952000006
Biosafety cabinet, class II type A2		
BRAND Disposable Plastic Cuvettes, Polystyrene	VWR International	47744-642
Chenille Kraft Flat Wood Toothpicks	VWR International	500029-808
cycloheximide	Research Products International	C81040-5.0
Dibasic potassium phosphate anhydrous, ACS grade	Fisher Scientific	P288-500
Dimethylformamide		
Dissecting microscope (Magnification of at least 10x)		
Ethanol - 190 Proof		
Falcon polystyrene 96 well microplates, flat-bottom	Fisher Scientific	08-772-3
Glass Alcohol Burner Wick	Fisher Scientific	S41898A / No. W-125
Glass Alcohol Burners	Fisher Scientific	S41898 / No. BO125
Glycerol ACS reagent	VWR International	EMGX0185-5
Kimberly-Clark™ Kimtech Science™ Kimwipes™ Delicate Task Wipers	Fisher Scientific	06-666-A
Magnesium chloride, ACS grade	VWR International	97061-356
Magnesium sulfate heptahydrate, ACS grade	VWR International	97062-130
Microcentrifuge tubes, 1.5 mL		
Microcentrifuge tubes, 2.2 mL		
Mini Beadbeater-96, 115 volt	Bio Spec Products Inc.	1001
Murashige & Skoog, Basal Salts	Caisson Laboratories, Inc.	MSP01-50LT
Pipet-Lite XLS LTS 8-CH Pipet 20-200uL	Rainin	L8-200XLS
Pipet-Lite XLS LTS 8-CH Pipet 2-20uL	Rainin	L8-20XLS
Polystyrene 100mm x 25mm sterile petri dish	VWR International	89107-632
Polystyrene 150mm x 15mm sterile petri dish	Fisher Scientific	FB08-757-14
Polystyrene 150x15mm sterile petri dish	Fisher Scientific	08-757-148
Pure Bright Germicidal Ultra Bleach 5.7% Available Chlorine (defined as 100% bleach)	Staples	1013131

Rifampicin	Gold Biotechnology	R-120-25
Silwet L-77 (non-ionic organosilicone surfactant co-polymer $C_{13}H_{34}O_4Si_3$ surfactant)	Fisher Scientific	NCO138454
Tips LTS 20 µL 960/10 GPS-L10	Rainin	17005091
Tips LTS 250 µL 960/10 GPS-L250	Rainin	17005093
VWR dissecting forceps fine tip, 4.5"	VWR International	82027-386

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RESPONSE TO THE EDITORIAL COMMENTS

Editorial Comment #1:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response to Editorial Comment #1:
We have thoroughly proof-read the manuscript.

Editorial Comment #2:
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Response to Editorial Comment #2:
We have formatted it as specified above.

Editor's Comment #3:
3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: (Caisson Laboratories, 113 Inc.), nanopure H2O, 3M Micropore, Silwet L-77 (Lehle Seeds), Eppendorf tubes, Mini-Beadbeater207 96, Bio Spec Products Inc., Kimwipes (Kimberly-Clark Worldwide, 218 Inc.), etc.

Response to Editorial Comment #3:
We have removed all commercial language from the manuscript and have referenced any commercial products in the Table of Materials and Reagents.

Editorial comment #4:
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
Rewording sentences to remove "should be" and "would be":

Response to Editorial Comment #4:

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We have reviewed the text in the protocol section and reworded the text so that it is written in the imperative tense. We have removed uses of "could be," "should be," and "would be". We added a minimal number of notes, and moved text to the discussion section.

Editorial comment #5:
5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response to Editorial Comment #5:
We have renumbered the protocol according to the instructions.

Editorial comment #6:
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step

Response to Editorial Comment #6:
We have reworked the protocol to simplify and ensure that individual steps only contain 2-3 actions per step.

Editorial comment #7:
7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response to Editorial Comment #7:
We have added more details to the protocol steps to elaborate on how the steps are performed.

Editorial comment #8:
8. 3.1, 3.4, 3.5, 3.6, 7: Please make substeps.

Response to Editorial Comment #8:
We have separated these steps into multiple substeps and moved some text to the discussion.

Editorial comment #9:
9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. We need at least 1 page of highlight for making a video. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and is in alignment with the title of the manuscript.

Response to Editorial Comment #9:
We have highlighted ~2.5 pages of text for filming.

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Editorial comment #10:
10. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Response to Editorial Comment #10:
We have moved this text after the Representative Results.

Editorial comment #11:
11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response to Editorial Comment #11:
We have not reused any figures from a previous publication. All figures and images are from independent replicates.

Editorial comment #12:
12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response to Editorial Comment #12:
We have reorganized the discussion section and added details that were previously in the protocol to the discussion section. We followed the general outline above.

Editorial comment #13:
13. Please do not abbreviate the journal titles in the references section.

Response to Editorial Comment #13:
We changed these to their full titles and reformatted the references as per JoVE instructions for authors.

Editorial comment #14:
14. Please sort the materials table in alphabetical order.

Response to Editorial Comment #14:
We sorted the materials table in alphabetical order.

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DEPARTMENT OF PLANT AND MICROBIAL BIOLOGY

RESPONSE TO THE REVIEWERS' COMMENTS

Reviewer #1 General Comments:

Manuscript Summary:
A simple, yet very useful, assay is described for assessing the response of tomato to Pseudomonas syringae pv. tomato (Pst). The introduction provides the appropriate background to set the context for the protocol (but see one comment below). The protocol itself is described in great detail and should be easy for any lab to follow whether or not they have previous experience with bacterial culture and/or inoculation. The figures are clear and will be especially helpful for researchers who are new to the tomato-Pst pathosystem. Overall, the seedling flood assay will be useful for many labs that study bacterial-plant interactions (not just the tomato-Pst system).

Major Concerns:
None

Response to Reviewer #1, General Comments:
We thank the reviewer for their kind words on our work.

Reviewer #1 Comment #1:
Minor Concerns:
Line 80-81: It is not correct that PstDC3000 has been the primary disease-causing strain (in the field). This strain is the primary one used for experimental work in the laboratory. The predominant strains in the field have typically been race 0 strains though most have not been characterized in any detail. As the authors state, race 1 strains are now becoming more common.

Response to Reviewer #1 Comment #1:
We thank the reviewer for pointing out this inaccuracy. We have rephrased this sentence and it now reads:

> Predominant strains in the field have typically been *Pst* race 0 strains that express either or both type III effectors AvrPto and AvrPtoB. DC3000 (*Pst*DC3000) is a representative race 0 strain and a model pathogen that can cause bacterial speck in tomato.

Reviewer #2 General Comments:

Manuscript Summary:
Hassan et al. described a graphical and written protocol to evaluate the bacterial pathogen growth and disease resistance in tomato plants. Those assays are critical to understand the plant-pathogen interaction and dissect the pathogenesis mechanism when plant accessions are challenged with different pathogen strains. This manuscript is well written and easy to follow. However, the authors need to address the following concerns on the manuscript:

Response to Reviewer #2 General Comments:
We thank the reviewer for their kind words on our work.

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Reviewer #2 Comment #1:

Major Concerns:

In section 5 -7, each plant was sampled at day 4 for pathogen growth data and then assayed for resistance phenotype after day 7-14. The sampling event during day 4 clearly introduces a wounding effect, which would stimulate Jasmonic acid pathway and counteract with the salicylic acid pathway. Thus, the pathogen growth data at day 4 may not fully correlates with the resistance phenotype due to the variation of the wounding effect. Highly recommend the author to use 2 sets of plants for pathogen growth and resistance phenotype analysis.

Response to Reviewer #2 Comment #1:

We thank the reviewer for this comment. For genotypically homogenous populations, we agree that it is best to use two different sets of plants for phenotyping and bacterial growth assays. However wild populations can be quite genetically diverse. For these populations, we prefer to perform qualitative and quantitative assessments of resistance on the same plant to ensure that we are identifying resistant plants. As the reviewer mentions, wounding could promote disease. However since we are screening for resistance, we would still enrich for individuals with strong immunity. We added the following text to step 8.11: "Alternatively, perform phenotypic analysis and bacterial growth assays on separate sets of plants."

Reviewer #2 Comment #2:

Minor Concerns:

1. Lack of definition for some abbreviations, e.g. AvrPto, AvrPtoB, Pto/Prf.

Response to Reviewer #2 Comment #2:

We further explained AvrPto and AvrPtoB in response to reviewer #1. Please see Reviewer #1 Comment #1 for the new text.

We added further information on *Pto/Prf* and this reads as follows:

> To combat bacterial speck disease, breeders introgressed the *Pto* [*P. syringae pv. tomato*]*/Prf* [*Pto resistance and fenthion sensitivity*] gene cluster from the wild tomato species *Solanum pimpinellifolium*, into modern cultivars[8,9]. The *Pto* gene encodes a serine-threonine protein kinase which together with *Prf*, an NLR, confer resistance to *Pst*DC3000 via recognition of effectors AvrPto and AvrPtoB.

Reviewer #2 Comment #3:

2. Personal pronoun should be avoided throughout the text.

Response to Reviewer #2 Comment #3:

We have removed all personal pronouns.

Reviewer #2 Comment #4:

3. A note/discussion on how to avoid tissue culture contaminations should be included since it is the fundamental part of this method.

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Response to Reviewer #2 Comment #4:
We added a new section on "Preparation and use of the biosafety cabinet."

Reviewer #2 Comment #5:
4. During the protocol section, method description sometimes is mixed up with notes/discussion. Highly recommend the author to revisit the protocol section and adjust those areas for a better reading experience for the audience.

Response to Reviewer #2 Comment #5:
We have rewritten the protocol section so that it focuses on the method. We added a minimal number of notes and moved notes/discussion to the discussion.

Reviewer #2 Comment #6:
5. In line 90-92, author briefly stated the limitation of using adult tomato pathogen assay, are there more methods that are available to address the plant-pathogen interaction in tomato? If so, an overview of those methods and brief explanation of the pros and cons of each method would be a nice info for the introduction section.

Response to Reviewer #2 Comment #6:
We added additional text to the discussion to describe plant-pathogen screens in tomato and to highlight benefits and drawbacks compared to the seedling flood assay.

Reviewer #2 Comment #7:
In lane 117, the authors recommend "The ideal media depth range for flood experiments ranged between 35mL and 45mL", but this statement is confusing the depth and volume concept.

Response to Reviewer #2 Comment #7:
We have changed this to volume instead of depth. It now reads:

> Media volume above 45 mL or below 35 mL may affect consistent death of susceptible controls as the volume may impact the surrounding microenvironment of the seedlings on the plate.

Reviewer #2 Comment #8:
4. In line 133, how does the author keep the plates in the 4˚C? Vertical? Inverted?

Response to Reviewer #2 Comment #8:
We added the following text to make this more clear. It now reads: "Ensure that plates are stacked flat and face up, so that the seeds do not shift on the plate."

Reviewer #2 Comment #9:
5. In line 147-155, author stated that "Patch bacteria (ie. PstT1) from a glycerol stock onto King's B (KB) media

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with appropriate antibiotics". Would the authors recommend the audience to isolate single colony on the Agar KB plates before re-streaking into the fresh KB media? The type of medium, solid or liquid is not clearly stated and could be confusing to the audience. Since this is method publication, a recipe and procedure to prepare the King's B medium would be helpful to guide the audience. In addition, how long would the authors keep using the "recently patched glycerol stock"?

Response to Reviewer #2 Comment #9:
We have clarified that the glycerol stock is made from a single colony. We added in KB broth or agar, and added a new section on "Preparation of King's B (KB) media". We added in details on how long to keep the patched glycerol stock.

Reviewer #2 Comment #10:
6. In line 157-158, the authors recommend operating on benchtop near lit ethanol burner, why not operate under the biosafety cabinet as shown in line 166?

Response to Reviewer #2 Comment #10:
We have reworded this section to indicate that bacteria should be aseptically resuspended.

Reviewer #2 Comment #11:
7. Text in Line 167-171 are confusing on how the serial dilution is made for different inoculum.

Response to Reviewer #2 Comment #11:
We have made two new sections to explain how the inocula are made. One section focuses on *Pst*T1 and the other on *Pst*DC3000 (Sections 6 and 7).

Reviewer #2 Comment #12:
8. The fact that optimal Silwet L-77 concentration in reference 22 was done by using in 10-day-old seedlings should be included in the statement in line 174-175.

Response to Reviewer #2 Comment #12:
We moved the details on surfactant optimization to the discussion and added in that the concentration was optimized for 10-day-old seedlings.

Reviewer #2 Comment #13:
9. Text in line 186 to 191 did not reveal some critical information: Does the final inoculum include the Silwet L-77?

Response to Reviewer #2 Comment #13:
We clarified that the surfactant is added to the final serial dilution only for each inoculum. This information is now included for each section on the two different inocula.

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Reviewer #2 Comment #14:
Does this flooding need to operate under the biosafety cabinet?

Response to Reviewer #2 Comment #14:
We added in more details to make it clear that flooding should occur in the biosafety cabinet.

Reviewer #2 Comment #15:
Does the timing (morning or afternoon) of the inoculation need to consistent across different pathogen infection experiments?

Response to Reviewer #2 Comment #15:
This is a good point. We added another sentence in the discussion to clarify. It reads:

> In addition, since plant-pathogen interactions can be affected by the circadian clock[24-26], it is recommended to infect the plants at a consistent time of the day.

Reviewer #2 Comment #16:
10. Could the author confirm that 3M Tape Micropore 1/2" x 10 YD CS 240 (1.25 cm x 9.1 m) was used in the Figure 1 and Figure 5? The resolution of the figures makes it looks like parafilm was used to seal the plates.

Response to Reviewer #2 Comment #16:
Micropore tape was indeed used to wrap the plates after flooding. The surgical tape and cover of the petri dish were removed to take photographs of the plates in Figures 1 and 5. As per the editor's comment and the journal's Instructions for Authors (Style Guidelines), we have deleted the commercial reference to "3M Micropore" and have referred to this as surgical tape.

Reviewer #2 Comment #17:
11. In Figure 2, the left and right panel showed different layout of genotypes. Would recommend the author to make it consistent across the figure 2.

Response to Reviewer #2 Comment #17:
We have amended the figure to include both genotypes on each panel.

Reviewer #3 General Comments:
Manuscript Summary:
The article describes and efficient method to identify resistance in tomato seedlings. The method is well-described and efficacy was demonstrated using two Pseudomonas syringae strains.
Major Concerns:
None.

Response to Reviewer #3 General Comments:
We thank the reviewer for their comments.

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Reviewer #3 Comment #1:
Minor Concerns:
On lines 124-126, the authors mention use of a "sterile biosafety cabinet". Can a biosafety cabinet be sterile? Should the authors perhaps mention that the biosafety cabinet should be properly disinfected?

Response to Reviewer #3 Comment #1:
We thank the reviewer for this comment and have added a new section on "Preparation and use of the biosafety cabinet."

Reviewer #3 Comment #2:
For lines 157-158, is it necessary to indicate that the bacteria should be resuspended near a lit burner? Perhaps re-word to indicate aseptic technique should be used. "Aseptically resuspend bacteria in sterile.....

Response to Reviewer #3 Comment #2:
We have reworded this section to indicate that bacteria should be aseptically resuspended.

Reviewer #3 Comment #3
Lines 166-171 were difficult to follow. As written it is unclear why one needs to make a 3/4 dilution and a 1/2 dilution. Please clarify.

Response to Reviewer #3 Comment #3:
The optimized dilutions for each strain were empirically determined in a previous publication (Hassan et al 2017). To clarify the procedure, we made two new sections: "Preparation of *Pst*T1 inoculum" (Section 6) and "Preparation of *Pst*DC3000 inoculum" (Section 7). See also response to Reviewer #2 Comment #11.

Reviewer #3 Comment #4:
For section 3.6, Should one add the Silwet to the last dilution? Or is Silwet added to the initial dilution and then carried through the dilutions?

Response to Reviewer #3 Comment #4:
We clarified that the surfactant is added to the final serial dilution only for each inoculum. This information is now included for each section on the two different inocula. See also the response to Reviewer #2 Comment #13.

Reviewer #3 Comment #5:
For section 4.2, instead of saying "until 3 min are up", maybe "for a total of 3 minutes".

Response to Reviewer #3 Comment #5:
Thank you, we have amended the sentence as suggested.

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Reviewer #3 Comment #6
Section 5.4: instead of on bench top near flame, maybe say "aseptically".

Response to Reviewer #3 Comment #6:
Thank you, we have amended the sentence as suggested.

Reviewer #3 Comment #7
For section 6.3, it is not clear after how long one should wait to count the colonies. Is there a time frame> Also, maybe clarify instead of saying so they fo not merge together, say before they merge together?

Response to Reviewer #3 Comment #7:
We have added step 10.4 and a brief note to elaborate on this point. It now reads:

> 4. Incubate at 28˚C for 36 hours, then visualize (Figure 2B) colonies on plates using a dissecting microscope to determine if colonies are large enough to count.
>
> NOTE: If the colonies are not large enough, re-incubate the plates and recheck the size of the colonies every few hours. Typically, colonies are countable ~36-48 hr after incubation.

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