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A Fast and Easy Method to Study *Ralstonia solanacearum* Virulence upon Transient Gene Expression or Gene Silencing in *Nicotiana benthamiana* Leaves

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[Abstract] *Ralstonia solanacearum* is a devastating soil-borne bacterial pathogen that causes disease in multiple host plants worldwide. Typical assays to measure virulence of *R. solanacearum* in laboratory conditions rely on soil-drenching inoculation followed by observation and scoring of disease symptoms. Here, we describe a novel inoculation protocol to analyze the replication of *R. solanacearum* upon infiltration into the leaves of *Nicotiana benthamiana,* in which gene expression has been altered using *Agrobacterium tumefaciens*. The protocol includes five major steps: 1) growth of *N. benthamiana* plants; 2) infiltration of *A. tumefaciens*; 3) *R. solanacearum* inoculation; 4) sample collection and bacterial quantitation; 5) data analysis and representation. The transient gene expression or gene silencing prior to *R. solanacearum* inoculation provides a straightforward way to perform genetic analysis of plant functions involved in the interaction between pathogen and host, using the appropriate combination of *A. tumefaciens* and *R. solanacearum* strains, with high sensitivity and accuracy provided by the quantitation of bacterial numbers in plant tissues.

Keywords: *Ralstonia solanacearum*, *Nicotiana benthamiana*, Virulence, Infection, Inoculation, Infiltration

[Background] *Ralstonia solanacearum* is considered one of the most destructive bacterial pathogens in plants worldwide due to its aggressiveness and wide host range. *R. solanacearum* invades host plants through their roots and colonizes their xylem vessels, finally spreading through the whole plant and leading to bacteria wilt disease (Xue *et al*., 2020). *R. solanacearum* is able to infect more than 250 plant species and is particularly well-adapted to infecting crops from the *Solanaceae* family, including tomato, potato, tobacco, eggplant, and other economically important crops (Mansfield *et al*., 2012). Experimental assays to assess the virulence of *R. solanacearum* in *Solanaceae* plants under laboratory conditions mostly rely on soil-drenching inoculation with a bacterial suspension followed by observation of disease symptoms (Morel *et al*., 2018). Other methods are based on the injection or infiltration of the bacterial suspension directly into the stems or leaves, bypassing the root penetration process and allowing a more sensitive and accurate quantitation of bacterial numbers in plant tissues (Morel *et al.*, 2018). These methods have been extensively used to compare the virulence of different *R. solanacearum* strains in *Solanaceae* plants; however, the analysis of different plant genotypes relies on the availability of relevant plant mutants or transgenic plants, or otherwise requires their generation, which is usually time-

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consuming. We recently developed a method to generate tomato plants with transgenic roots suitable for subsequent soil-drenching inoculation with *R. solanacearum*, which allows for the faster generation of transgenic root material to study root invasion and the subsequent generation of disease symptoms (Morcillo *et al.*, 2020). However, this method does not allow for accurate quantitation of bacterial replication in plant tissues. *Nicotiana benthamiana*, a model plant from the *Solanaceae* family, is well known for its suitability for transient gene expression mediated by *Agrobacterium tumefaciens* (Li, 2011). Therefore, we envisioned a straightforward procedure to manipulate gene expression in *N. benthamiana* tissues using *A. tumefaciens* followed by the subsequent infiltration of *R. solanacearum* and quantitation of *R. solanacearum* replication over time. Such a method should overcome two major issues: (i) many *R. solanacearum* strains, including the reference GMI1000 strain, are not virulent in *N. benthamiana* due to the recognition of two effector proteins (AvrAA and RipP1) by the immune system of this plant species (Poueymiro *et al.*, 2009); and (ii) the inoculated *R. solanacearum* strain should be differentiated from the *A. tumefaciens* population previously infiltrated into the same leaf tissues. To overcome the first issue, we used the *R. solanacearum* Y45 strain, which was originally isolated from tobacco plants (Li *et al.*, 2011) and lacks both AvrAA and RipP1, allowing it to replicate effectively in *N. benthamiana* leaf tissue (Sang *et al.*, 2020). To circumvent the second issue, we transformed *R. solanacearum* Y45 with a plasmid that confers resistance to the antibiotic tetracycline to allow the selection of *R. solanacearum* Y45 colonies in solid medium while avoiding growth of *A. tumefaciens*. This simple method can be used to accurately determine *R. solanacearum* virulence in *N. benthamiana* tissues expressing heterologous genes (Sang *et al.*, 2020; Wei *et al.*, 2020), overexpressing endogenous genes, or undergoing gene silencing mediated by RNAi. The versatility of this method allows the study of bacterial virulence factors in plant tissues by expressing them in plant cells prior to bacterial inoculation, as well as genetic analysis of the contribution of plant genes to disease resistance or susceptibility.

Materials and Reagents

1. Conical centrifuge tubes (50 ml) (GeneBrick, catalog number: GP0-7500)
2. Microcentrifuge tubes (1.5 ml) (BBI, catalog number: F600620-0001)
3. Needleless syringes (1 ml)
4. Plastic Petri dishes (90 mm diameter)
5. Pipette tips (AIBIO, catalog number: T1040000)
6. Paper towels
7. Cork borer (Integra Miltex, model: 33-34-P/25)
8. (Optional) Sprial counting grid (90 mm diameter) (Interscience, Easysprial®, catalog number: 413014)
9. Metal beads
10. Spectrophotometer plastic cuvettes (BRAND, catalog number: 759015)
11. Syringe filters (0.45 µm) (Millex, Millex®-HV, catalog number: SLHV033RB)
12. Distilled sterile water

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13. 75% ethanol (Sinopharm Chemical Reagent, SCR®, catalog number: 801769610)
14. Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, catalog number: T8877-5G)
15. Glucose (Sinopharm Chemical Reagent, SCR®, catalog number: 63005518)
16. Bacto peptone (BD, catalog number: 211677)
17. Yeast extract (OXOID, catalog number: LP0021)
18. Casein hydrolysate (Casamino acids) (Sigma-Aldrich, catalog number: 22090-500G)
19. Agar (Sinopharm Chemical Reagent, SCR®, catalog number: 10000561)
20. Tryptone (OXOID, catalog number: LP0042)
21. Sodium chloride (NaCl) (Sinopharm Chemical Reagent, SCR®, catalog number: 10019318)
22. 3',5'-Dimethoxy-4'-hydroxyacetophenone (AS) (Sigma-Aldrich, catalog number: 2478-38-8)
23. Magnesium chloride hexahydrate ($MgCl_2·6H_2O$) (Sinopharm Chemical Reagent, SCR®, catalog number: 10012818)
24. Potassium hydroxide (KOH) (Sinopharm Chemical Reagent, SCR®, catalog number: 10017018)
25. MES free acid monohydrate (MES) (Amresco, catalog number: Amresco E169)
26. Dimethyl sulfoxide (DMSO) (Diamond, catalog number: A100231-0500)
27. Standard potting soil (Pindstrup, catalog number: 1034593214)
28. Tetracycline hydrochloride (Sangon Biotech, catalog number: T0422)
29. Vermiculite
30. Phi medium (see Recipes)
31. 1% (W/V) TTC solution (see Recipes)
32. 20% (W/V) glucose solution (see Recipes)
33. Agrobacterium infiltration buffer (see Recipes)
34. LB medium (see Recipes)
35. Tetracycline hydrochloride (optional, depending on the antibiotic resistance of the strains used) (see Recipes)

Equipment

1. Tweezers
2. Scissors
3. Medium- and high-throughput tissue grinder (QIAGEN, model: Tissue Lyser II)
4. NanoDrop spectrophotometer (Thermo Scientific, model: NanoDrop 2000c)
5. Water distiller/sterilizer (Millipore, model: Mili-Q intergral 10L)
6. Spiral plater (optional) (Interscience, Easysprial®, catalog number: 412000)
7. Vortex (Scientific Industries, model: Vortex-Genie 2, catalog number: S1-0246)
8. (Optional) Alcohol lamp
9. Petri dish incubator at 28°C (Panasonic, model: MIR-262-PC)
10. Autoclave (SANYO, model: MLS-3780)
11. Flow hood (clean bench) (Shanghaishangjing, model: CA-1390-1)

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12. Plant growth chamber (Percival, model: I-36VL)
13. Electronic balance (Sartorius, model: BSA224S)
14. Centrifuge (Eppendorf, model: centrifuge 5424)
15. pH meter (Sartorius, model: PB-10)
16. Tube incubator (shaker) at 28°C (Eppendorf, New Brunswick™, catalog number: m1324-0006)

Software

1. GraphPad Prism 7 (GraphPad, http://www.graphpad.com), or equivalent program for statistical analysis
2. Microsoft Excel (Microsoft), or equivalent spreadsheet calculation program

Procedure

A. Growing *Nicotiana benthamiana* plants
   1. Sow *N. benthamiana* seeds in a 1:1 mix of potting soil and vermiculite and place in a growth room at 25°C and 65% relative humidity under a 16-h light/8-h dark photoperiod with a light intensity of 130 mE $m^{-2} \cdot s^{-1}$.
   2. Transfer 9-day-old seedlings into individual pots and allow them to grow under the same growth conditions until they are 4-5 weeks old (Figure 1).

Figure 1. Growth of *Nicotiana benthamiana* seedlings in soil. Red arrows indicate leaves suitable for infiltration.

B. Transient expression using *Agrobacterium tumefaciens* (hereafter, Agrobacterium)
   Note: This is a standard procedure detailed in Li (2011).
   1. This part of the protocol is optional to perform transient gene expression or gene silencing prior to *R. solanacearum* inoculation. If this step is not needed, proceed to Section C.
   2. Prepare and autoclave LB medium with and without agar. Allow LB medium with agar to cool until the bottle can be touched with bare hands (but is still in liquid form), add the appropriate antibiotics, and pour the medium into Petri dishes under a sterile flow hood.
   3. Streak out the Agrobacterium strains carrying the desired plasmids (see Note 1) onto solid LB medium and incubate at 28°C for 24 h. Incubate for a longer time if bacterial colonies need to be isolated.

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4. Collect Agrobacterium biomass directly from the Petri dish using a sterile pipette tip and resuspend in 1 ml infiltration buffer. Dilute 10-fold and measure OD_{600} using a spectrophotometer.
5. Prepare an Agrobacterium suspension with a final concentration of OD_{600} = 0.2-0.5 in infiltration buffer.
6. Select fully expanded leaves of 4-5-week-old *N. benthamiana* plants for infiltration (Figure 2). Using a permanent marker pen, draw a circle on each half of the leaf to control the area for infiltration. On the abaxial side of the leaf, prick softly at the center of the circle with a needle (*e.g.*, from a 1-ml syringe).

   Note: Infiltration time: it is better to inoculate R. solanacearum in the morning to obtain reproducible and robust infection results. Therefore, it is advised to infiltrate Agrobacterium in the morning and leave 24 hours as a suitable time for heterologous gene expression before R. solanacearum inoculation. For RNAi-mediated silencing, we leave 3-9 days between Agrobacterium and R. solanacearum inoculation, depending on the silencing efficiency of each construct (see Note 1).

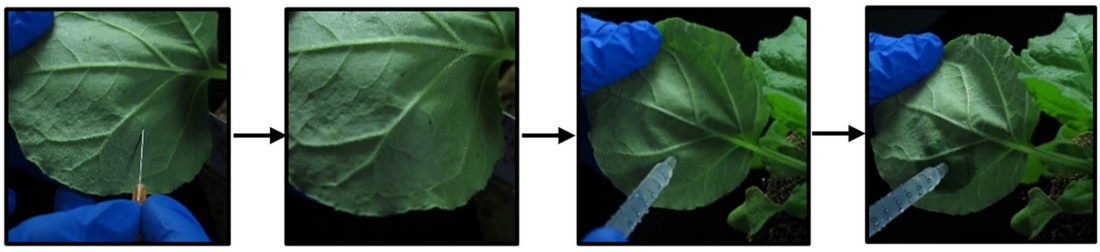

Figure 2. Illustration of the infiltration step

7. Infiltrate the Agrobacterium suspension carefully into the marked area.
   a. To this end, place a finger on the adaxial side of the leaf and place the needleless syringe at the same spot on the abaxial side (avoid spots with excessive protuberant veins).
   b. Use a 90° angle to avoid excessive spillage of the Agrobacterium suspension, but press gently on the leaf to avoid tissue damage.
   c. Slowly press the plunger until the Agrobacterium suspension starts filling the plant tissue, which becomes visibly darker upon soaking. Agrobacterium containing the plasmid for gene expression/silencing and the control plasmid can be infiltrated side-by-side in the same leaf to avoid leaf-to-leaf variation (see notes).
   d. Wipe the wet surface of the leaf gently with tissue paper and correct the circles (if necessary) with the actual infiltrated area.

   Notes:

   i. Infiltration of samples/controls: the different Agrobacterium strains used for transient gene expression/silencing and the control strain can be infiltrated in each half of the same leaf to avoid leaf-to-leaf variation. In such cases, bacterial numbers can also be analyzed as a ratio

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of the bacterial growth in both halves of the leaf. However, it is important to note that, if the transiently expressed gene/protein induces systemic responses, these could also affect the other half of the leaf. Therefore, the choice should be made depending on the specific nature of the transiently expressed gene.

ii. *Infiltration of N. benthamiana leaves works better in dry leaves without water droplets from condensation or contact with other leaves. Some practice is advised (choice of infiltrated area, position of hands, pressure over the leaf tissue and the plunger, etc.) to optimize leaf infiltration before the actual experiment.*

8. Place the plants back into the growth chamber at 25°C and 65% relative humidity under a 16-h light/8-h dark photoperiod with a light intensity of 130 mE $m^{-2} \cdot s^{-1}$.

C. Inoculation with *Ralstonia solanacearum* Y45 (Figure 3)

1. Select a *R. solanacearum* strain that is pathogenic in *N. benthamiana* (*e.g.*, Y45; [Li *et al*., 2011; Sang *et al*., 2020]). If Agrobacterium was previously infiltrated into plant tissues, the *R. solanacearum* strain used should be resistant to an antibiotic that allows the differentiation of Y45 from the existing Agrobacteria at the time of sampling (see Note 2).
2. Prepare and autoclave phi medium with and without agar. Allow the phi medium with agar to cool until the bottle can be touched with bare hands (but is still in liquid form), then add 5 ml/L 1% TTC stock solution and 25 ml/L 20% glucose stock solution.
3. Streak out Y45 onto solid phi medium and incubate at 28°C for 2 days.
4. Select pink colonies and pick using a sterile pipette tip to inoculate 10 ml phi liquid medium in a 50-ml conical centrifuge tube. Incubate the cultures on a shaker at 28°C overnight.
5. Centrifuge the suspensions for 5 min at 4,000 × *g*. Remove the supernatant and resuspend the bacterial pellet in 10 ml sterile distilled water. Measure OD_{600} using a spectrophotometer. A suspension in distilled water with an OD_{600} = 1 contains approximately 10^9 cfu/ml (Morel *et al.*, 2018).

   Note: In our experience, resuspending the bacteria in sterile distilled water (or other solution, such as $MgCl_2$) does not affect the viability or virulence of R. solanacearum.

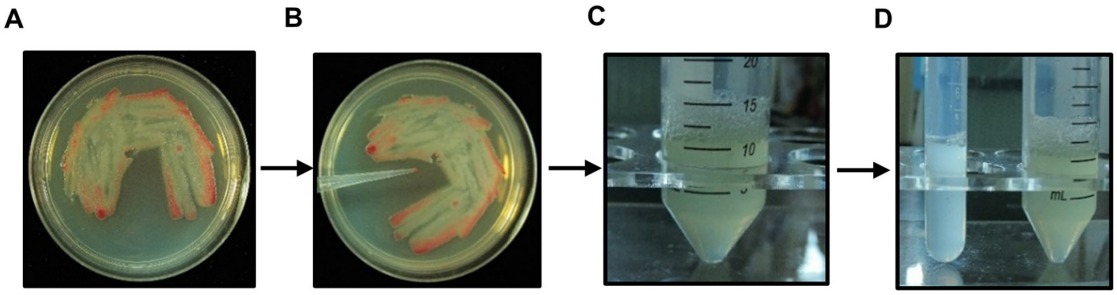

Figure 3. *Ralstonia solanacearum* inoculation and collection. A. Bacteria growing on solid phi medium with TTC. B. Collection of bacterial biomass from pink colonies using a pipette tip. C. Bacterial culture after a 14-h overnight incubation (OD_{600} = 1.08). D. Bacterial suspension in

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sterile distilled water (left).

6. Prepare a suspension of Y45 at the final desired concentration, depending on your requirements, by performing serial dilutions using sterile distilled water. Consider the possibility of testing the virulence of different doses of your specific strain under your specific experimental conditions. Under our conditions, we selected 10^5 cfu/ml as a suitable concentration that allows reproducible bacterial replication without causing the development of necrotic symptoms during the experimental period (Figure 4).

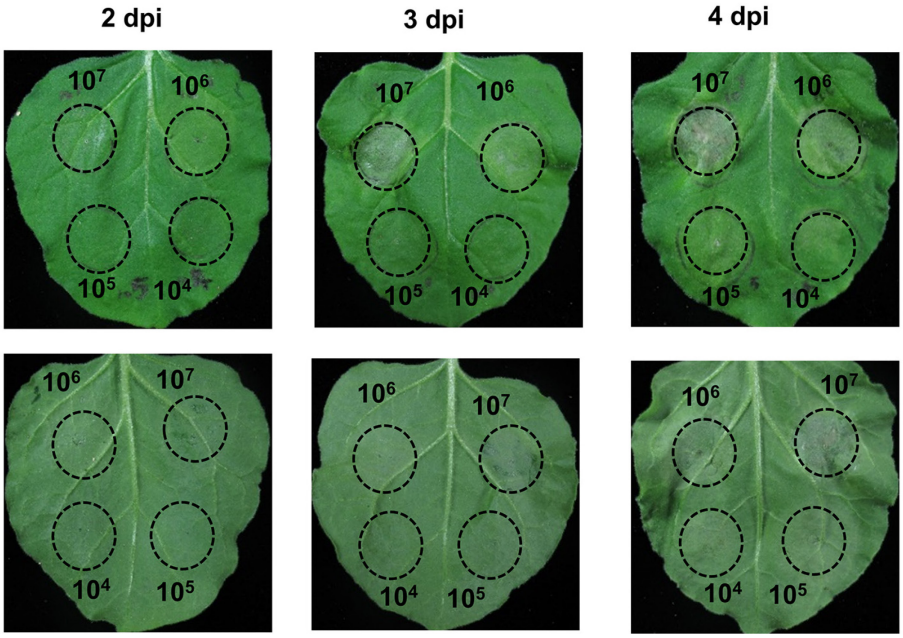

Figure 4. Symptoms of *N. benthamiana* tissues inoculated with different doses of Y45 at the indicated days post-inoculation (dpi) with *R. solanacearum*. Upper images show the adaxial sides, and lower images show the abaxial sides.

7. Select fully expanded leaves of 4-5-week-old *N. benthamiana* plants for infiltration (Figure 1). If Agrobacterium was previously infiltrated (Section B; see note at the Step B6 regarding infiltration time), infiltrate Y45 into the marked circles using a 1-ml needleless syringe as previously performed for Agrobacterium (Figure 2). Dry the surface of the leaves using tissue paper to remove residual bacterial inoculum from the leaf surface and place the inoculated plants in the growth chamber until sample collection.
   Note: After Y45 infiltration, we usually place plants in a different growth chamber at 27°C and 75% relative humidity since R. solanacearum infection is more efficient and reproducible at a higher temperature and humidity; however, a specific test experiment could be conducted to determine the efficiency and reproducibility of infection depending on the availability of growth chambers under these conditions.

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D. Sample collection

1. Prepare 1.5-ml microcentrifuge tubes and measure their weight using an electronic balance.
2. Cut the inoculated leaves using scissors and place them in a clean Petri dish containing sterile distilled water. Immerse both sides of the leaf in water for 5 s to wash the leaf surface and remove the surface water by placing the leaf on clean tissue paper.
3. Collect 4 leaf discs (4 mm diameter) from each infiltrated area (avoiding areas with potential damage from the infiltration step) using a sterile cork borer and transfer the discs to 1.5-ml microcentrifuge tubes (Figure 5). Measure the weight of the tubes again.

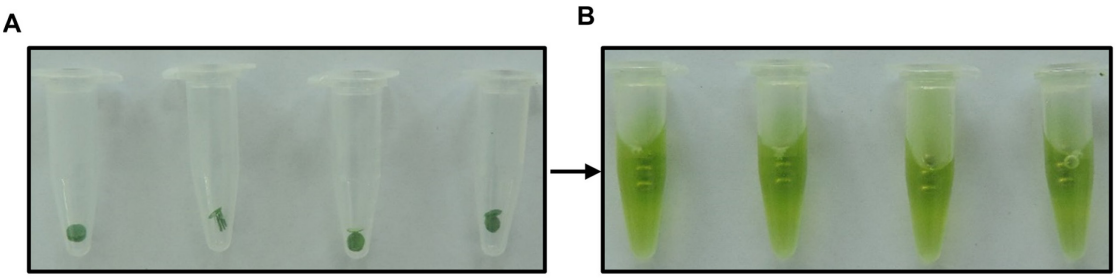

Figure 5. Collection of samples. A. Four leaf discs collected in 1.5-ml tubes using a 4-mm diameter cork borer. B. Homogenized plant tissue in 1 ml sterile water.

4. Add 3 small metal beads to each tube using tweezers, followed by 100 µl sterile distilled water. Grind plant tissue using a tissue grinder at a frequency of 25 oscillations/s for 2 min.
5. Add 900 µl sterile distilled water to the homogenized tissue and mix well using a vortex (Figure 5). Perform serial dilutions of the homogenized tissue by placing 100 µl in 900 µl sterile distilled water. Remember to change pipette tips between each dilution to avoid carryover of bacteria from higher dilutions.
6. Spread 50 µl appropriate dilutions onto solid phi medium plates containing the appropriate antibiotic to differentiate Y45 colonies from the existing Agrobacteria in the homogenized tissue (see Notes). See the legend of Figure 6 for an estimation of the dilutions plated at each time point. Spreading onto plates can be performed manually using a sterile spreader, sterile plastic beads, or the bottom of a sterile 1.5-ml microcentrifuge tube, or using a spiral plater (optional, see notes) (Figure 6).

   Notes:

   a. *Spreading colonies onto plates: disinfect hands with 75% alcohol before plating, and work under a sterile flow hood. An alcohol lamp can be used to avoid contamination if working on a bench. If plates are too wet after spreading the sample dilutions, they can be subsequently dried under a flow hood to obtain clear single colonies.*
   b. *Spiral plater: if available, a spiral plating machine can be used to spread bacterial dilutions. This is particularly useful for large-scale experiments, where numerous samples are required. There is no significant difference in the results obtained by plating manually and those obtained using the machine, as shown in Figure 6A and 6B.*

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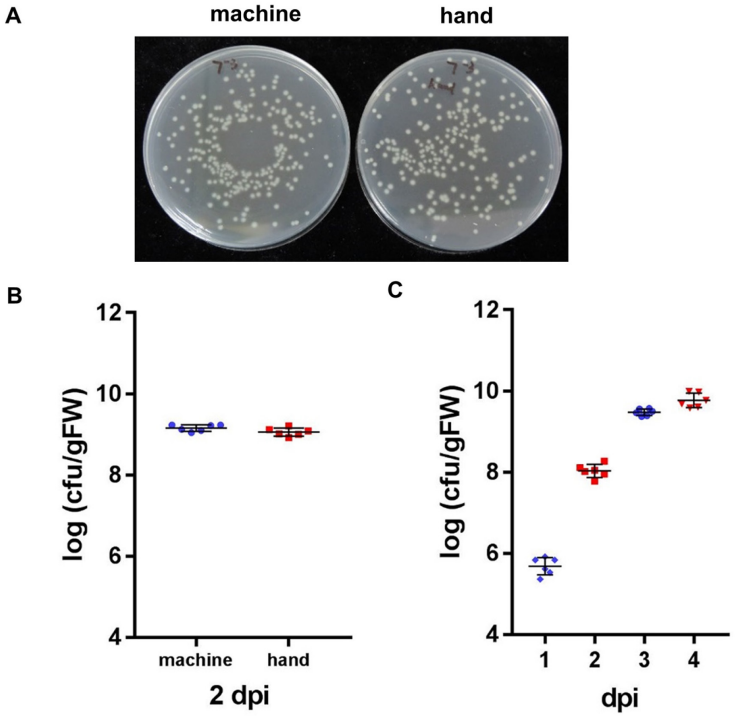

Figure 6. *Ralstonia solanacearum* Y45 growth. A. Colonies obtained on solid phi medium plates using the same bacterial dilution, by manual spreading or using the spiral plating machine. B. Comparison of cfu numbers using manual spreading or the spiral plating machine. C. Time-course experiment to determine Y45 growth. *N. benthamiana* leaves were first inoculated with Agrobacterium to express GFP (used as a control protein in our assays); one day later, a 10^5 cfu/ml inoculum of Y45 was infiltrated into the same leaves. Samples were then collected at 1, 2, 3, and 4 days post-inoculation (dpi) with Y45, as indicated. To count these numbers, 10^0 (undiluted) and 10^{-1} dilutions were plated 1 dpi, 10^{-1}, and 10^{-3} dilutions were plated 2 dpi, 10^{-3} and 10^{-4} dilutions were plated 3 dpi, and 10^{-4} and 10^{-5} dilutions were plated 4 dpi.

7. Incubate the plates upright (see Notes) at 28°C for 2 days. Count the colonies and process the data.
   Note: Incubation of R. solanacearum after sampling: colonies of R. solanacearum are very mucoid; therefore, plates should be incubated upright to avoid bacterial secretions dripping.

Data analysis

To calculate the number of bacteria by plant fresh weight, import the tube weight, full weight, dilution factor, and number of cfu (colony-forming units) into an Excel spreadsheet. Enter the sector of the plate when counting colonies plated using a spiral plater. Table 1 shows examples of the calculations performed when plating using the spiral plater and manually.

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Table 1. Calculation of bacterial numbers in plant tissues

	A	B	C	D	E	F	G	H	I	J	K	L
1		tube weight/g	full weight/g	fresh weight/mg	dilution	cfu	sector	volume(μl)	cfu/ml	dilution factor	cfu/g FW	Log (cfu/g FW)
2	Machine	1.0113	1.0178	6.5	2	108	1	1	108000	100	1661538462	9.220510399
3	Machine	1.0185	1.0262	7.7	2	131	1	1	131000	100	1701298701	9.23078057
4	Machine	0.9693	0.9771	7.8	2	133	1	1	133000	100	1705128205	9.231757038
5	Machine	0.9877	0.997	9.3	2	125	1	1	125000	100	1344086022	9.128427064
6	Machine	0.9773	0.9883	11	2	122	1	1	122000	100	1109090909	9.044967146
7	Machine	0.9879	0.9967	8.8	2	111	1	1	111000	100	1261363636	9.100840307
8	Manual	1.0113	1.0178	6.5	3	428	Full	50	8560	1000	1316923077	9.119560408
9	Manual	1.0185	1.0262	7.7	3	623	Full	50	12460	1000	1618181818	9.209027317
10	Manual	0.9693	0.9771	7.8	3	408	Full	50	8160	1000	1046153846	9.019595556
11	Manual	0.9877	0.997	9.3	3	564	Full	50	11280	1000	1212903226	9.083826151
12	Manual	0.9773	0.9883	11	3	465	Full	50	9300	1000	845454545.5	8.927090263
13	Manual	0.9879	0.9967	8.8	3	444	Full	50	8880	1000	1009090909	9.003930294

Calculation steps:

These calculations correspond to cfu numbers obtained by manual plating. If plating using a spiral plater, follow the manufacturer's instructions to calculate bacterial numbers.

1. Obtain the fresh weight of the plant tissue using the formula =(C8-B8)*1,000 and change the units from g to mg for subsequent calculation.
2. Calculate bacterial numbers (cfu/ml) using the formula (F8/H8)*1,000, and change the volume units from µl to ml.
3. Column E indicates the dilution series. For example, E8 indicates that bacteria were counted after two serial dilutions; therefore, the dilution factor (J8 = 10^E8) is 1,000.
4. To refer bacterial numbers to plant fresh weight, the weight units can be changed again from mg to g, and the final product can be calculated using the following formula: K8 = PRODUCT(I8, 1,000, J8, 1/D8), resulting in 1316923077 cfu/gFW. This value can be converted for representation in a logarithmic scale on the base of 10 (row L).
5. Input the resulting final data into GraphPad Prism7 or a similar program for statistical analysis. To compare results individually, preform a Student's *t*-test and present the mean value, standard error, and p value to indicate the statistical significance of the differences.

Notes

1. Choice of Agrobacterium strains: we usually use the Agrobacterium GV3101 strain carrying plasmids from the pGWB series (Nakagawa *et al.*, 2007a) to express tagged proteins in *N. benthamiana*. When GFP fusion proteins are used, we use free GFP as a control protein (Nakagawa *et al.*, 2007b) [as in Figure 2B of Sang *et al.* (2020)]. To perform transient gene silencing, we use an RNAi approach with pK7GWIWG2-II vectors (Karimi *et al.*, 2002). Gene fragments targeting specific genes using RNAi can be easily designed using diverse online tools (https://vigs.solgenomics.net/; http://plantgrn.noble.org/pssRNAit/). We use Agrobacterium carrying an empty vector as a negative control. It is important to always verify gene overexpression as well as silencing efficiency by performing RNA extraction and RT-qPCR.
2. Choice of *R. solanacearum* strain: to allow for the differentiation of Y45 from the existing Agrobacteria at the time of sampling, we transformed the Y45 wild type strain with a pRCT-derivative plasmid (Monteiro *et al.*, 2012) using natural transformation (Coupat *et al.*, 2008). This plasmid confers resistance to tetracycline; therefore, we add tetracycline to the phi medium used to plate bacteria after sampling.
3. Preparation of plates: after pouring the medium into Petri dishes, keep the plates uncovered under the flow hood for approximately 40 min when plating using the spiral plater or for around 20 min when plating manually. The spiral plater requires the plates to be drier in order to prevent the different circles from contacting each other; on the other hand, manual plating is more uniform if the plates are more humid. Sterile water can be added to the plates before manual plating if they are too dry.

4. Data representation: representation of the bacterial numbers (or cfu) can be performed relative to the fresh weight (as indicated in our protocol) or relative to leaf area, using the size of the cork borer as a reference. In the latter case, there is no need to measure the weight of the tubes before and after sampling.
5. Waste disposal: autoclave all the material used in this experiment before disposal to avoid release of *R. solanacearum* into the environment. Clean the work bench and flow hood with 75% alcohol after finishing the experiment.

Recipes

1. Phi medium
   10 g Bacto peptone
   1 g Yeast extract
   1 g Casamino acids
   Add water to 1 L.
   To prepare solid medium, add 15 g agar to 1 L medium and autoclave.
2. 1% (W/V) TTC solution
   Dissolve TTC in distilled water and filter the solution with a 0.45-µm syringe filter to avoid contamination.
   Note: Store the 1% TTC solution at room temperature or 4°C in a dark environment.
3. 20% (W/V) glucose solution
   Glucose should be dissolved in distilled water and autoclaved. Store this stock solution at 4°C.
4. Agrobacterium infiltration buffer
   1 M MES 100 µl
   1 M $MgCl_2$ 100 µl
   150 mM AS 10 µl
   Add 9.890 ml water to reach 10 ml.
   Adjust MES to pH 5.7 with KOH.
   Dissolve MES and $MgCl_2$ in sterile water and filter the solutions with a 0.45-µm syringe filter to avoid contamination.
   Store the solution at 4°C.
   Dissolve AS in DMSO and store the stock solution at -20°C.
5. LB medium
   10 g Tryptone
   5 g NaCl
   5 g Yeast extract
   Adjust pH to 7.5 with KOH, and add water to 1 L.
   To prepare solid medium, add 15 g agar to 1 L medium and autoclave.
6. Tetracycline (optional, depending on the resistance of the strains used)

Prepare a stock solution of tetracycline hydrochloride (10 mg/ml) in sterile distilled water (working in a flow hood) and store at -20°C. The final concentration in the medium should be 10 µg/ml.

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Competing interests

The authors have no competing interests to declare.

Ethics

No human or animal subjects are used in this protocol.

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