

Tomato Stem Injection for the Precise Assessment of *Ralstonia solanacearum* Fitness in *Planta*

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[Abstract] *Ralstonia solanacearum* is a soil-borne pathogen with worldwide distribution that causes bacterial wilt disease in more than 250 plant species. *R. solanacearum* invades plants through the roots, reaches the vascular system, and colonizes the whole plant by moving through the xylem, where it eventually replicates rapidly, causing plant death. Usual assays to measure the virulence of *R. solanacearum* under laboratory conditions rely on soil-drenching inoculation followed by observation and scoring of disease symptoms. Here, we describe a protocol to assess the replication of *R. solanacearum* following injection into tomato stems. This protocol includes four major steps: 1) growth of tomato plants; 2) *R. solanacearum* injection into tomato stems; 3) collection of tomato xylem samples and bacterial quantitation; and 4) data analysis and representation. This method bypasses the natural penetration process of the pathogen, thus minimizing variation associated with stochastic events during bacterial invasion, and provides a sensitive and accurate measurement of bacterial fitness inside xylem vessels.

Keywords: *Ralstonia solanacearum*, Stem injection, Tomato, Virulence, Fitness, Bacterial wilt

[Background] *Ralstonia solanacearum* is the causal agent of bacterial wilt disease in more than 250 plant species including important crops, such as tomato, potato, pepper, and eggplant, among others, and is considered one of the most dangerous plant pathogens in the world (Mansfield *et al.*, 2012). *R. solanacearum* is a soil-borne pathogen that enters plants through the roots, using wounds, root tips, and secondary root emergence points as penetration sites; it then progresses via the root cortex, finally reaching the vascular system (Xue *et al.*, 2020). In susceptible plants, *R. solanacearum* moves through the xylem vessels to colonize the whole plant; eventual replication may lead to populations up to 10¹⁰ bacteria per gram of plant tissue in diseased plants, which will block water and nutrient flow in the vascular system and eventually cause plant wilting and death (Peeters *et al.*, 2013). Multiple experimental methods to assess *R. solanacearum* virulence in plants under laboratory conditions have been described previously (Morel *et al.*, 2018). The most widely used assay relies on soil-drenching of plant roots with *R. solanacearum* suspensions, followed by observation of the resulting wilting symptoms over time (Morel *et al.*, 2018). Other assays involve bypassing the root penetration process by introducing the bacterial suspensions directly into plant tissues (either leaves or stems) and provide more accurate assessments of bacterial replication (Macho *et al.*, 2010; Morel *et al.*, 2018; Yu and

Macho, 2021).

In this protocol, we provide a detailed description of a method aimed at quantitating bacterial fitness in tomato xylem vessels by direct injection into the stem using a microsyringe; given the subsequent fast bacterial replication and plant colonization, we assume that a substantial proportion of this inoculum reaches the xylem vessels. At the desired time after inoculation, samples are taken from 2 cm above the inoculation site by collecting xylem sap from an excised stem section. Bacterial quantitation in this xylem sap provides a clean measurement of bacterial fitness in the plant vascular system. Like other Gram-negative bacterial pathogens, *R. solanacearum* requires a type III secretion system to inject type III effector (T3E) proteins into host cells and cause disease. Since T3E activities often contribute redundantly to the development of disease, it is generally difficult to determine their involvement in virulence; however, we have recently used this method to detect the virulence attenuation of different *R. solanacearum* knockout mutants lacking individual effectors (Xian *et al.*, 2020; Yu *et al.*, 2020) and to analyze bacterial pathogenicity following chemical treatment of plant tissues (Wang *et al.*, 2021), revealing a simple, versatile, and powerful assay for the study of *R. solanacearum*-plant interactions.

Materials and Reagents

1. Microcentrifuge tubes (1.5 ml and 2 ml) (BBI, catalog number: F600620-0001)
2. Pipette tips
3. Plastic Petri dishes (90 mm diameter)
4. Jiffy pots (41 mm diameter) (Jiffy International, Kristiansand, Norway)
5. Spectrophotometer plastic cuvettes (BRAND, catalog number: 759015)
6. Syringe filter (0.45 µm) (Millex, Millex®-HV, catalog number: SLHV033RB)
7. Tomato seeds (*Solanum lycopersicum* cv. Moneymaker)
8. Standard potting soil (Pindstrup, catalog number: 1034593214)
9. Vermiculite (generic)
10. Distilled sterile water
11. 75% ethanol (Sinopharm Chemical Reagent Co, SCR®, catalog number: 801769610)
12. Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, catalog number: T8877-5G)
13. Glucose (Sinopharm Chemical Reagent Co, SCR®, catalog number: 63005518)
14. Bacto peptone (BD, catalog number: 211677)
15. Yeast extract (OXOID, catalog number: LP0021)
16. Casein hydrolysate (Casamino acids) (Sigma-Aldrich, catalog number: 22090-500G)
17. Agar (Sinopharm Chemical Reagent Co, SCR®, catalog number: 10000561)
18. Phi medium (see Recipes)
19. 1% (W/V) TTC solution (see Recipes)
20. 20% (W/V) glucose solution (see Recipes)

Equipment

1. Micro sample syringe 10 μ l (Sangon Biotech, catalog number: E718LD0023)
2. Sterile scalpel
3. EasySpiral[®] Automatic Serial Diluter and Plater (optional) (Interscience, Easyspiral[®], catalog number: 412000)
4. Plant growth chamber (Percival, model: I-36VL)
5. Autoclave (SANYO, model: MLS-3780)
6. Centrifuge (Eppendorf, model: Centrifuge 5424)
7. pH meter (Sartorius, model: PB-10)
8. Petri dish incubator at 28°C (Panasonic, model: MIR-262-PC)
9. Tube incubator (shaker) at 28°C (Eppendorf, New Brunswick[™], catalog number: m1324-0006)
10. NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 2000c)
11. Water distiller/sterilizer (Millipore, model: Milli-Q integral 10L)
12. Vortex (Scientific Industries, model: Vortex-Genie 2, catalog number: S1-0246)

Software

1. GraphPad Prism 7 (<https://www.graphpad.com/scientific-software/prism/>)

Procedure

A. Growing tomato plants

1. Germinate tomato seeds (*Solanum lycopersicum* cv. Moneymaker) in a 1:2 mixture of standard potting soil and vermiculite. Grow seedlings for one week in a growth chamber (25°C, 16 h light/8 h dark photoperiod, 130 mE m⁻² s⁻¹, 65% humidity) (Figure 1A).
2. Soak Jiffy pots thoroughly with tap water without fertilizer (an excess of fertilizer may cause stress to the tomato seedlings) (see Note 1). Transfer tomato seedlings to individual water-soaked jiffy pots containing a pre-packed soil mixture (Figure 1B) (see Note 1). Grow tomato plants in the same growth chamber for another three weeks (Figure 1C) (see Note 2). Approximately 6-8 plants per treatment are recommended to obtain robust results.



Figure 1. Growth of tomato seedlings in Jiffy pots. A. Germination of tomato seeds (*Solanum lycopersicum* cv. MoneyMaker) in a 1:2 mixture of standard potting soil and vermiculite. B. Transferring tomato seedlings to individual water-soaked jiffy pots containing a pre-packed soil mixture. C. Four-week-old tomato plants ready for *Ralstonia* inoculation.

B. Preparation of *R. solanacearum* inoculum

1. Prepare solid and liquid phi medium (see Recipes) following standard procedures (Yu and Macho, 2021).
2. Streak out *R. solanacearum* strains on solid phi medium plates containing TTC and glucose (see Recipes; Yu and Macho, 2021) and prepare a bacterial suspension as previously described (Yu and Macho, 2021).
3. Dilute the final bacterial suspension in distilled water to an $OD_{600} = 0.1$, which corresponds to approximately 10^8 CFU/ml (Morel *et al.*, 2018). Perform serial dilutions of the bacterial suspension using distilled water to reach 10^6 CFU/ml in a volume of 1 ml in a sterile 2-ml microcentrifuge tube (see Note 3) (Figure 2). Consider the possibility of testing the virulence of different doses of your specific strains under your specific experimental conditions. Under our conditions, we selected 10^6 CFU/ml as a suitable concentration to allow gradual but reproducible bacterial replication inside tomato stems during the first 3 days post-inoculation (dpi) without causing the development of necrotic symptoms during the experiment (Figure 3).

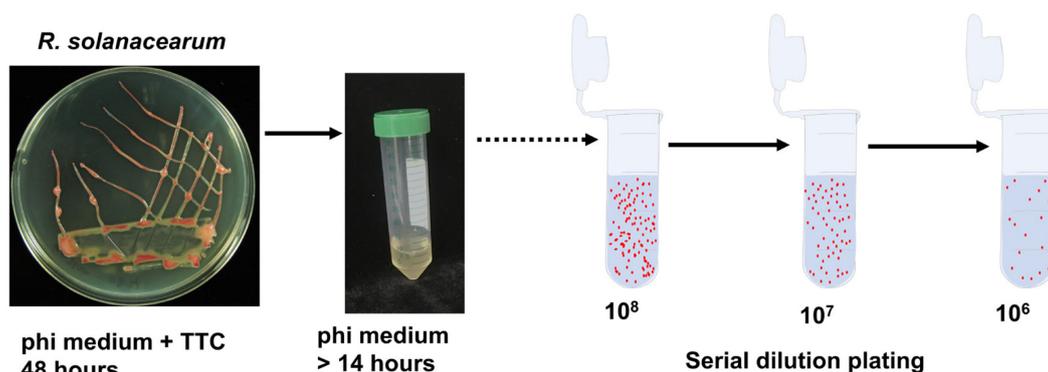


Figure 2. Preparation of *R. solanacearum* inoculum

C. Inoculation (stem injection) and sampling of xylem sap

1. Using a 10- μ l microsyringe, inject 5 μ l bacterial suspension into the stems of 4-week-old tomato plants (see Note 4). The inoculation site should be approximately 2 cm below the cotyledon emergence site (Figure 3A and 3B). When performing stem injection, use an angle of approximately 30° between the syringe and the stem to facilitate injection of the bacterial suspension.
2. Place the inoculated tomato plants in a growth chamber (28°C, 12 h light/12 h dark, 130 mE m⁻² s⁻¹, 75% humidity) for 36-72 h until subsequent sampling at the appropriate time points.
3. Transect the stem with a sterile scalpel just below the cotyledon emergence site (Figure 3C).
4. Using two fingers, gently squeeze the lower part of the tomato stem to allow the xylem sap to emerge and then collect 2.5 μ l xylem sap using a 2.5- μ l pipette (see Note 5) (Figure 3D).

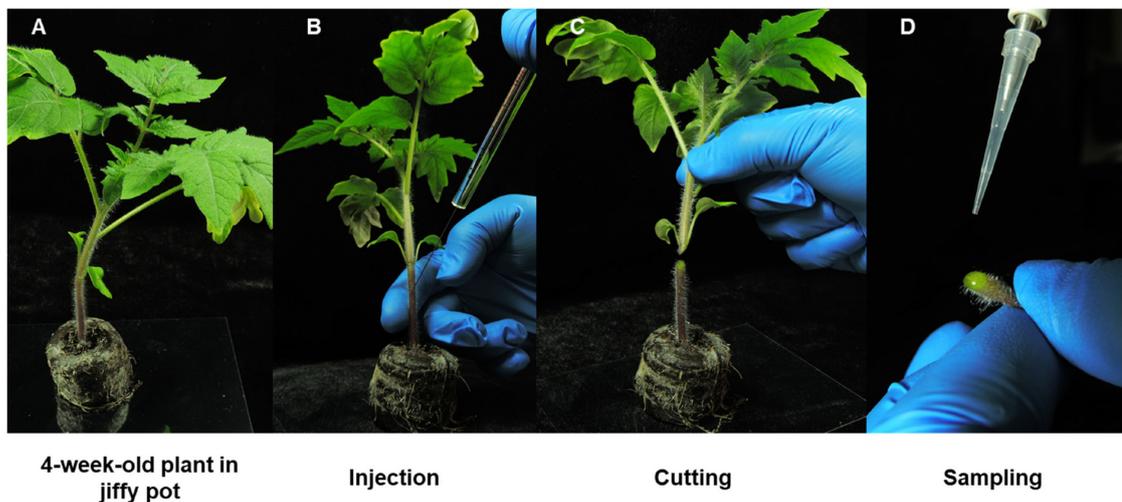


Figure 3. Stem injection and sampling of xylem sap. A. Four-week-old tomato plants in Jiffy pots. B. Using a 10- μ l microsyringe, inject 5 μ l bacterial suspension into the stems of 4-week-old tomato plants. C. Transect the tomato stems with a sterile scalpel just below the cotyledon emergence site. D. Collect 2.5 μ l xylem sap at the cutting site using a micropipette.

D. Bacterial quantitation

1. Dissolve the xylem sap in 1 ml sterile water in a sterile 1.5-ml microcentrifuge tube (Figure 4A).
2. Dilute and spread bacteria (50 μ l) on phi medium plates using standard procedures, as previously described (Yu and Macho, 2021). Spreading can be performed manually using a sterile spreader, plastic beads, or the bottom of a 1.5-ml microcentrifuge tube, or using a spiral plater (Yu and Macho, 2021).
3. Incubate the plates upright at 28°C for 2 days. Count the colonies and process the data (Figure 4B) (see Note 6).

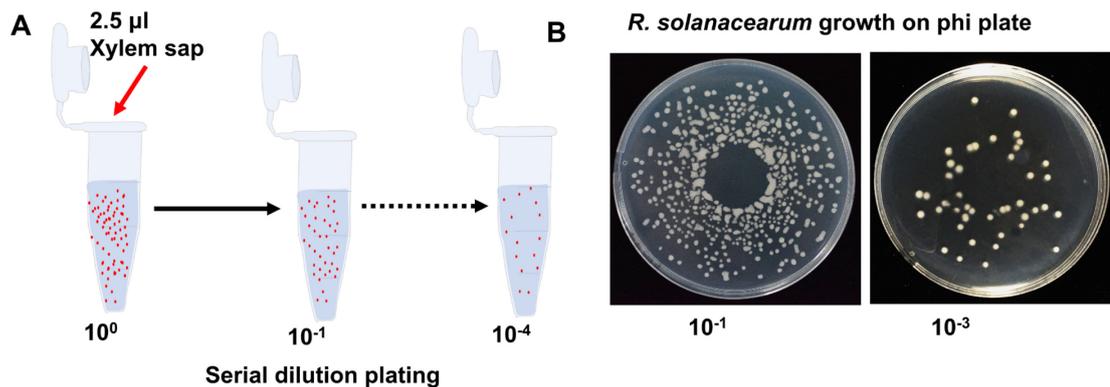


Figure 4. Preparation of serial dilutions and bacterial spreading. A. Dissolve the xylem sap containing *Ralstonia* in 1 ml sterile water in a sterile 1.5-ml microcentrifuge tube; dilute the suspensions by 10 to 10^4 times. B. Spread the diluted bacterial suspensions (we tried to spread 10^{-1} or 10^{-3}) on phi medium plates using standard procedures, as previously described (Yu and Macho, 2021). Incubate the plates upright at 28°C for 2 days and count the colonies on solid phi medium plates. Photos in B show the usual colony pattern after plating using a spiral plater (Yu and Macho, 2021).

Data analysis

1. Using the number of colonies on each readable plate, calculate bacterial numbers in the sampled xylem sap. If plating was performed manually, apply the following formula:
$$R. \text{ solanacearum numbers in xylem sap (CFU/ml)} = \text{colony number} \times 20 \times 10^3 \times 10^{(\text{dilution factor})/2.5}$$

Note: "Colony number": number of colonies on the readable plate. "20" corresponds to the result of dividing 1000 µl final diluted bacteria between 50 µl bacterial solution spread on the plate. "10³" corresponds to the conversion of CFU/µl to CFU/ml. "2.5" corresponds to the 2.5 µl xylem sap taken as a sample and dissolved in 1 ml water. "10^(dilution factor)" corresponds to the additional serial dilution of the xylem sap.
2. If plating was performed using a spiral plater, follow additional calculations as previously described (Yu and Macho, 2021).
3. Repeat the experiment at least 3 times. Input the final data to GraphPad Prism 7 or a similar program for statistical analysis. To compare results individually, perform a Student's *t*-test and report the mean value, SEM, and *P* value to indicate the statistical significance of the differences. Results are better displayed using a logarithmic scale and the individual data points together with the mean \pm SEM (Figure 5).

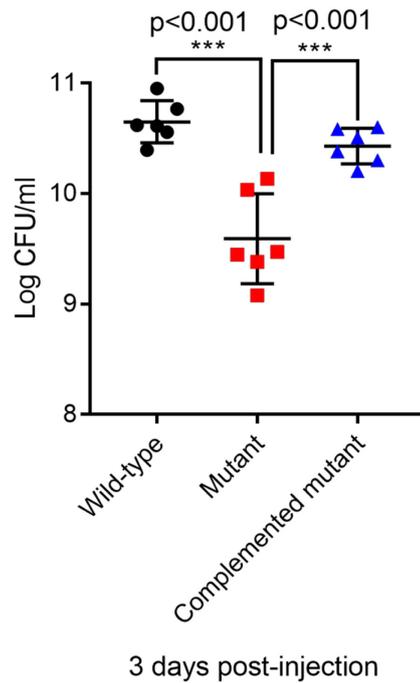


Figure 5. Representation of the growth of *R. solanacearum* GMI1000 wild-type, mutant, and complemented strain in tomato stems. Different colors represent different strains, and horizontal bars represent mean values (n = 6 plants per strain). Data are represented as the mean ± SEM. Asterisks indicate significant differences ($***P < 0.0001$, *t*-test). The graph was plotted using GraphPad Prism 7.

Notes

1. If the tap water has a high mineral content or contains excessive impurities, purified water can also be used.
2. Grow no more than 24 tomato plants in each tray (29 × 20 cm), and separate the plants to avoid intertwined roots among individual plants. Water the plants normally once every two days, and keep the jiffy pots wet.
3. For serial dilution, use a 1:10 dilution for each step.
4. Optionally, move the tomato plants to a growth chamber (28°C, 12 h light/12 h dark, 130 mE m⁻² s⁻¹, 75% humidity) 12 h before *R. solanacearum* injection to minimize interference caused by changing the growth chamber after inoculation. This does not make a difference under our conditions.
5. For tomato sampling, remove the oozing sap gently with sterile paper, and clamp the stem top with a finger to push the xylem sap out for sampling.
6. Waste disposal: Autoclave all the material used in this experiment before disposal to avoid releasing *R. solanacearum* into the environment. Clean the work bench and flow hood with 75%

alcohol after the experiment.

Recipes

1. Phi medium

10 g Bacto peptone

1 g Yeast extract

1 g Casamino acids

Add water to reach 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

Note: Store 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 at 4°C.

*Note: Add 1% TTC and 20% glucose when preparing solid Phi medium plates to induce pink *R. solanacearum* colonies.*

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Competing interests

The authors have no competing interests to declare.

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