Plant Pathogenicity Phenotyping of Ralstonia solanacearum Strains

Arry Morel, Nemo Peeters, Fabienne Vailleau, Patrick Barberis, Gaofey Jiang, Richard Berthomé, Alice Guidot

▶ **To cite this version:**

Arry Morel, Nemo Peeters, Fabienne Vailleau, Patrick Barberis, Gaofey Jiang, et al.. Plant Pathogenicity Phenotyping of Ralstonia solanacearum Strains. Host-Pathogen Interactions: Methods and Protocols, 1734, Carlos Medina,Francisco Javier López-Baena, 2018, Methods in Molecular Biology, 978-1-4939-7604-1; 978-1-4939-7603-4. 10.1007/978-1-4939-7604-1_18 . hal-02791793

HAL Id: hal-02791793
https://hal.inrae.fr/hal-02791793

Submitted on 22 Jul 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

Chapter 18

Plant Pathogenicity Phenotyping of *Ralstonia solanacearum* Strains

Arry Morel, Nemo Peeters, Fabienne Vailleau, Patrick Barberis, Gaofei Jiang, Richard Berthomé, and Alice Guidot

Abstract

In this chapter, we describe different methods for phenotyping strains or mutants of the bacterial wilt agent, *Ralstonia solanacearum*, on four different host plants: *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), tobacco (*Nicotiana benthamiana*), or *Medicago truncatula*. Methods for preparation of high volume or low volume inocula are first described. Then, we describe the procedures for inoculation of plants by soil drenching, stem injection or leaf infiltration, and scoring of the wilting symptoms development. Two methods for measurement of bacterial multiplication in planta are also proposed: (1) counting the bacterial colonies upon serial dilution plating and (2) determining the bacterial concentration using a qPCR approach. In this chapter, we also describe a competitive index assay to compare the fitness of two strains coinoculated in the same plant. Lastly, specific protocols describe in vitro and hydroponic inoculation procedures to follow disease development and bacterial multiplication in both the roots and aerial parts of the plant.

Key words *Ralstonia solanacearum*, Phenotyping on plants, In planta bacterial growth measurements, Tomato, *Nicotiana benthamiana*, *Arabidopsis thaliana*, *Medicago truncatula*

1 Introduction

Ralstonia solanacearum is the causal agent of bacterial wilt of more than 200 plant species and is responsible for one of the most devastating bacterial plant disease in the world [1]. *R. solanacearum* is a soilborne plant pathogen which naturally infects plants through root tips and lateral root cracks, invades the xylem vessels and spreads rapidly to aerial parts of the plant through the vascular system [2, 3]. In susceptible plants, *R. solanacearum* population can reach up to 10^{10} colony-forming units (CFU) per gram of fresh weight, inducing clogging of the vascular system and causing the wilting symptoms and eventual plant death [4].

Many pathogenicity determinants are required by *R. solanacearum* for successful infection of its hosts [4]. An

Carlos Medina and Francisco Javier López-Baena (eds.), *Host-Pathogen Interactions: Methods and Protocols*, Methods in Molecular Biology, vol. 1734, https://doi.org/10.1007/978-1-4939-7604-1_18, © Springer Science+Business Media, LLC 2018

essential pathogenicity determinant is the type 3 secretion system (T3SS) as the corresponding mutant strains produce no disease symptoms on plants and their multiplication in plant tissues is considerably reduced, up to 10^4-fold less than the wild-type strain [3]. The T3SS enables the translocation of type 3 effector (T3E) proteins into the plant cell [5]. A total of 74 T3Es have been identified in the sequenced strain GMI1000 [6]. Several of these T3Es are avirulence factors since they can induce plant defense response on some specific plant lines. This is the case for both RipP1 (formerly PopP1) and RipAA (formerly AvrA) on various *Nicotiana* spp. [7], for RipP1 on a petunia line [8] and for RipP2 (formerly PopP2) on some *Arabidopsis* ecotypes [9]. However, the contribution to the overall virulence of most of the T3Es is not clear as most single T3E mutant strains do not significantly differ from the wild-type strain. One notable exception is the strong contribution of RipG7 (formerly GALA7) for the virulence of *R. solanacearum* specifically on the model legume *Medicago truncatula* [2, 10, 11]. Furthermore, several multiple T3E mutants have been shown to be less aggressive than their wild-type strain, suggesting functional overlap between these T3Es [10, 12, 13].

The absence of a detectable difference between the virulence phenotype on plants of a mutant and a wild-type strain may reflect a lack of sensitivity of the experimental procedure used. A more sensitive assay to compare the phenotype on plants is to measure the capacity of the bacteria to multiply within the host plants. This can be achieved by counting the numbers of CFU in plant material upon serial dilution plating [13]. Determination of the bacterial multiplication in the plant material is also possible using a quantitative-PCR (qPCR) approach with *R. solanacearum* specific primers [9, 14]. More recently, a competitive index assay has been developed that allows quantifying a differential fitness of two *R. solanacearum* strains in planta [15]. In such assay, the wild-type and mutant strains are coinoculated in the same proportion but at very low concentration, in the same plant tissue. This inoculation method reduces the plant-to-plant variation and enhances thus the detection power of a differential fitness between the two strains. Using this approach, the contribution of several T3Es in *R. solanacearum* in planta fitness has been demonstrated, while previous experiments based on disease symptom development failed to reveal a role in virulence for these T3Es [15]. There are thus many methods for phenotyping *R. solanacearum* wild-type strains and mutants on plants. The method chosen depends on the objective of the experiment and on the required sensitivity to compare the phenotypes of several bacterial strains on different plants.

In this chapter, we describe seven different methods for phenotyping *R. solanacearum* strains and mutants on plants. It should be stressed that these methods can also be used to assay several

plant genotypes, variants or mutants with a wild-type *R. solanacearum* strain, to assess the contribution to susceptibility or resistance of specific plant genes. After description of the procedures for high volume and low volume inoculum preparation, this chapter describes methods for (1) inoculation of plants by soil drenching, (2) stem injection or (3) leaf infiltration and (4) measurement of bacterial multiplication in planta. It also describes (5) competitive index assays to compare the fitness in planta of two strains. Lastly, specific protocols are described to follow disease development and bacterial multiplication under (6) in vitro or (7) hydroponic conditions, two procedures that allow an access to the plant roots.

2 Materials

1. Glycerol stock of *R. solanacearum* wild-type strains or mutants stored at −80 °C.
2. Complete BG medium (for 1 L): 10 g Bacto peptone, 1 g Casamino acids, 1 g yeast extract. For solid BG medium, add 5 g glucose, 0.05 g triphenyltetrazolium chloride, and 15 g agar.
3. Antibiotics: Gentamycin (10 mg/L), Kanamycin (50 mg/L), Spectinomycin (40 mg/L), Tetracycline (10 mg/L). Half the antibiotic concentration for liquid cultures.
4. Fåhraeus medium [16]: Prepare stock solutions of macroelements and microelements. Make five solutions for macroelements (for 100 mL): 13.20 g $CaCl_2{\cdot}2H_2O$, 12 g $MgSO_4{\cdot}7H_2O$, 10 g KH_2PO_4, 7.5 g $Na_2HPO_4{\cdot}2H_2O$, and 0.5 g ammonium iron (III) citrate. Make five solutions at 1 mg/mL for each microelement: $MnCl_2{\cdot}4H_2O$, $CuSO_4{\cdot}5H_2O$, ZnCl, H_3BO_3, and $Na_2MoO_4{\cdot}2H_2O$. For 1 L of Fåhraeus medium: 1 mL of each stock solution of macroelements except for $Na_2HPO_4{\cdot}2H_2O$ (2 mL) and 100 µL of each stock solution of microelements; add 0.33 g/L $(NH_4)_2SO_2$; adjust to pH 7.5 and for solid medium incorporate 15 g Difco Agar before autoclaving.
5. Araponics medium: mix FloraGro and Floramicro solutions (General Hydroponics). For solid araponics medium, add 0.65% Difco Agar.
6. Plant materials:
   (a) 4-week-old tomato plants (*Solanum lycopersicum* cultivar Super Marmande VR) grown in a classic greenhouse, 1 plant per 7 × 7 cm pot or 16 plants per 30 × 30 cm tray containing 2 L potting soil (*see* **Note 1**).

(b) 4-week-old tobacco plants (*Nicotiana benthamiana*) grown in the green house in 7 × 7 cm pot, or 16 plants in a 30 × 30 cm tray.

(c) 4-week-old *Arabidopsis* plants (*Arabidopsis thaliana* Col-0 ecotype) grown in a growth chamber (22 °C, 70% humidity, 9 h light) in jiffy pots.

(d) 4-week-old *Arabidopsis* plants (*Arabidopsis thaliana* Col-0 ecotype) grown in hydroponic conditions (*see* Subheading 3) in a growth chamber (22 °C, 70% humidity, 9 h light).

(e) 10-day-old *Medicago truncatula* Gaertn. Genotype A17 (derived from cultivar Jemalong) grown in a growth chamber (22 °C, 70% humidity, 9 h light) in jiffy pots.

(f) 3-day-old *Medicago truncatula* Gaertn. Genotype A17 grown in square petri dishes (12 × 2 cm) containing Fåhraeus medium with an interface of CYG™ seed growth paper (Mega International, St. Paul, MN, U.S.A.) between plantlets and Fåhraeus agar medium.

7. Growth chamber.
8. 28 °C incubator.
9. Rotary shaker.
10. Spectrophotometer.
11. 7 mm diameter punch borer.
12. Disposable 1 mm diameter glass beads.
13. Ceramic beads.
14. 1 mL disposable syringes.
15. 25 µL Exmire microsyringe.
16. Mill grinder.
17. 13 cm aquarium air pump with a ceramic diffuser.
18. 10 L Plastic bins (Athena series, Fami storage system).
19. qPCR machine.
20. 95% anhydrous H_2SO_4.
21. 12% sodium hypochlorite.
22. 70% and absolute ethanol.
23. TRIzol reagent.
24. Chloroform–isoamyl alcohol (24:1).
25. 5 M NaCl.
26. RNAse free water.
27. Black 96-well plates (clear bottom).
28. Quant-iT Picogreen dsDNA Assay Kit (Thermo Fisher Scientific).

29. 2× SYBR Green master mix.
30. Scalpel and extra fine tip tweezers.

3 Methods

3.1 High Volume Inoculum Preparation

1. Under sterile conditions, streak out the strains on BG medium with the appropriate antibiotics from their −80 °C glycerol stock.
2. Incubate at 28 °C for 48 h.
3. Take an isolated colony and inoculate 50 mL liquid BG medium with the appropriate antibiotics in a 250 mL sterile Erlenmeyer flask.
4. Incubate at 28 °C under agitation at 180 rpm in a rotary shaker for more than 16 h.
5. Take 1 mL to measure the OD_{600nm} with a spectrophotometer.
6. Calculate the bacterial concentration, knowing that $OD_{600nm} = 1$ corresponds to 10^9 CFU/mL.
7. Dilute the bacterial suspension to 10^8, 5×10^7 or 10^7 CFU/mL. The dilution can be performed by mixing the original BG medium grown bacteria with water (for a large inoculum, room-temperature tap water can also be used).
8. Take 1 mL to check the dilution by measuring the OD_{600nm} (*see* **Note 2**).

3.2 Low Volume Inoculum Preparation

1. Under sterile conditions, streak out the strains on BG medium with appropriate antibiotics from their −80 °C glycerol-stock.
2. Incubate at 28 °C for 48 h.
3. Take an isolated colony and inoculate 10 mL liquid BG medium with the appropriate antibiotics in a 50 mL sterile Erlenmeyer flask.
4. Incubate at 28 °C under agitation at 180 rpm in a rotary shaker for more than 16 h.
5. Transfer 1.8 mL of the bacterial suspension in a 2 mL Eppendorf.
6. Centrifuge for 2 min at 13,000 rpm in a table top centrifuge.
7. Resuspend the pellet in 1.8 mL sterile water.
8. Take 1 mL to measure the OD_{600nm} with a spectrophotometer.
9. Calculate the bacterial concentration knowing that $OD_{600nm} = 1$ corresponds to 10^9 CFU/mL.
10. Dilute the bacterial suspension to 10^8 CFU/mL in a final volume of 1.8 mL.

11. Take 1 mL to check the dilution by measuring the OD_{600nm} which should be equal to 0.1 (*see* **Note 2**).
12. Depending on the bioassay considered, dilute the bacterial suspension to 10^6, 5×10^4 or 10^4 CFU/mL using the suspension dilution procedure in a final volume of 1 mL.

3.3 Soil Drenching Infection of *Arabidopsis* or *Medicago* Plants

1. Prepare a high volume inoculum (2 L per 50 plants to analyze) to a final concentration of 10^8 CFU/mL.
2. Place the *Arabidopsis*- or *Medicago*-containing jiffy pots in a large container to soak in the inoculum for 20 min (2 L of inoculum per 50 plants) (*see* **Note 3**).
3. Transfer the *Arabidopsis*- or *Medicago*-containing jiffy pots to a new tray (alternatively place on large sheets of disposable aluminum foil).
4. Pour enough fine potting soil onto the remaining inoculum in order to obtain a firm surface and place the plants back (*see* **Note 4**).
5. Incubate in the growth chamber for 10–20 days with the following conditions: 27 °C the day/26 °C the night, 80–85% relative humidity, 12 h light.
6. Once per day, score the wilting symptoms on a visual scale of 1–4 (Fig. 1) (*see* **Note 5**).
7. Repeat the experiment at least three times to draw conclusions (*see* **Note 6**).

3.4 Soil Drenching Infection of Tomato Plants and Measurement of Bacterial Multiplication In Planta

1. Transfer the tomato plants in the growth chamber (28 °C day/27 °C night, 80–85% relative humidity, 12 h light) 2 days before the inoculation.
2. Prepare a high volume inoculum (500 mL per strain to analyze, for a 16-plant tray or for ten individually potted plants) to a final concentration of 5×10^7 CFU/ mL (*see* **Note 7**).
3. Pour the inoculum onto the soil surrounding the tomato plants: 50 mL in pots with 1 plant or 500 mL in trays with 16 plants (*see* **Note 8**).
4. Incubate in the growth chamber for 10–20 days.
5. Once per day, score the wilting symptoms on a visual scale of 1–4 (Fig. 1) (*see* **Notes 5** and **6**).
6. Measure the bacterial multiplication in planta as follows: Sample 1 cm of the stem above the cotyledons and weight it (*see* **Note 9**). Sterilize the surface by putting it in 70% ethanol for 30 s, then rinse the stem in sterile water for 30 s (individual petri dishes can be used for the ethanol and water baths). Cut the stem in six parts (one centered longitudinal section and three transversal sections) with a sterile scalpel and transfer the

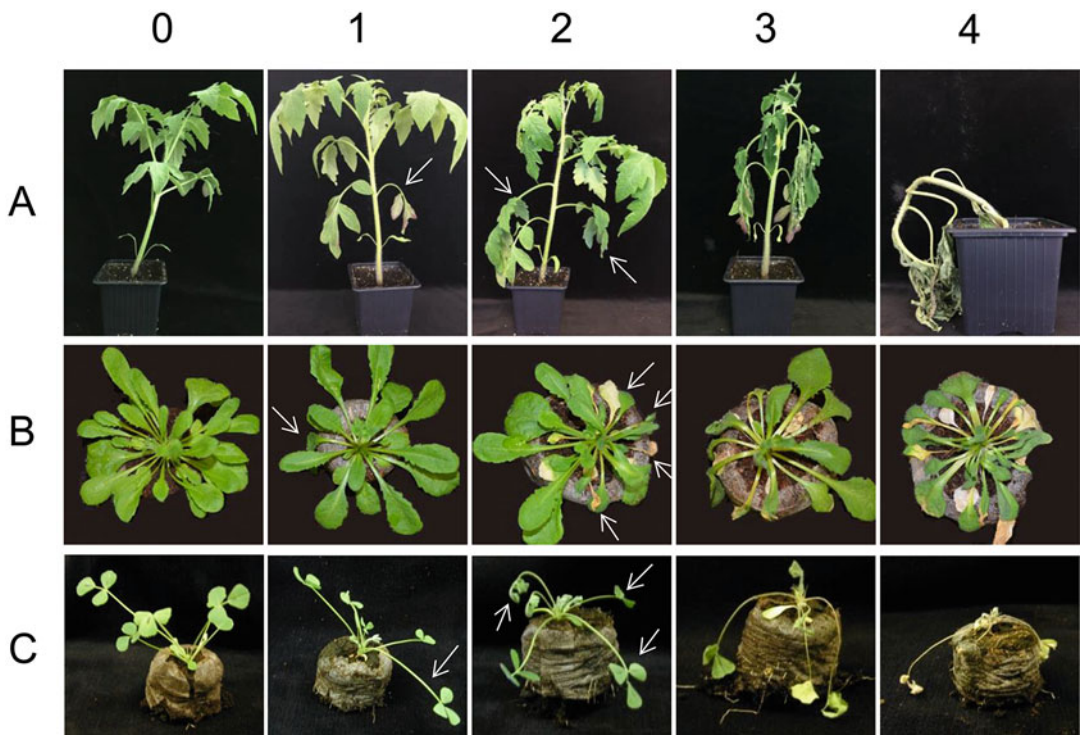

Fig. 1 Symptoms notation scale for (**a**) tomato (*Solanum lycopersicum*), (**b**) *Arabidopsis thaliana*, and (**c**) *Medicago truncatula*. (**c**) Symptoms notation scale is reprinted from Vailleau et al., 2007 [17], Characterization of the Interaction Between the Bacterial Wilt Pathogen *Ralstonia solanacearum* and the Model Legume Plant *Medicago truncatula*, Molecular Plant-Microbe Interactions Vol. 20: pp. 159–167. 0 = No wilting. 1 = First wilting symptoms appears. 2 = Wilting of half of the plant. 3 = Wilting of most of the plant. 4 = Complete wilting. White arrows show leaves that are wilting

sections into a 2 mL Eppendorf tube containing 1 mL sterile water. Incubate at room temperature for at least 30 min and less than 2 h so that the bacteria can diffuse from the stem sections into the sterile water. Generate serial 10× dilutions of the bacterial suspension and plate 100 µL of the appropriate dilutions on solid BG medium with antibiotics corresponding to the strain (*see* **Note 10**). Incubate at 28 °C for 48 h and count bacterial colonies. Calculate the bacterial concentration into the stem using the following formula:

Bacterial concentration (CFU/g fresh matter) = [(number of colonies × 10)/dilution factor]/weight of the sampled stem.

7. Repeat the experiment at least three times to draw conclusions, each experiment containing either one tray with 16 plants or 10 plants in individual pots. If the bacterial load is assessed over the course of the disease development rather than at the end of the wilting, randomly select individual tomato plants to assess their bacterial load.

3.5 Stem Injection of Tomato Plants and Measurement of Bacterial Multiplication In Planta

1. Transfer the tomato plants in the growth chamber (28 °C day/27 °C night, 80–85% relative humidity, 12 h light) 2 days before the inoculation.
2. Prepare a small volume inoculum (1 mL per ten plants to inject) to a final concentration of 10^6 CFU/mL.
3. Inject 10 μL of the bacterial suspension into the stem 0.5 cm above the cotyledons using a microsyringe. Between each strain or mutant, sterilize the microsyringe by pumping up and down 70% ethanol three times and then sterile water three times.
4. Incubate in the growth chamber for 6–10 days.
5. Once per day, score the wilting symptoms on a visual scale of 1–4 (Fig. 1) (*see* **Notes 5** and **6**).
6. Measure the bacterial multiplication in planta: sample 1 cm of the stem 1 cm above the inoculation point, weight it and follow the procedure to measure the bacterial multiplication in planta after soil drenching infection of tomato plants (*see* Subheading 3.4).
7. Repeat the experiment at least three times to draw the conclusions, each experiment containing either one tray of 16 plants or 10 plants in individual pots.

3.6 Assay for In Planta Growth of R. solanacearum by Leaf Infiltration of Nicotiana benthamiana Plants

1. Prepare a small volume inoculum (5 mL per plant to infiltrate) to a final concentration of 5×10^4 CFU/mL (*see* **Note 11**).
2. Chose a single leaf per plant and infiltrate the bacterial suspension with a blunt 1 mL disposable syringe (*see* **Note 12**). Gently wipe the excess inoculum from the abaxial and adaxial leaf surfaces.
3. Immediately after infiltration, sample four discs (7 mm diameter punch borer) from one half side of the inoculated leaf, in order to measure the original inoculum per square cm of leaf surface.
4. Incubate the plants in a growth chamber with the following conditions: 28 °C day/27 °C night, 80–85% relative humidity, 12 h light.
5. Put the leaf discs in a 2 mL Eppendorf tube and grind them with a fixed amount of disposable 1 mm diameter glass beads at 30 Hz for 30 s with a mill-grinder. Place the tubes in a centrifuge and do a short spin to pellet all plant debris stuck on the Eppendorf lids.
6. Add 1.8 mL sterile water, vortex and dilute it.
7. Plate the appropriate 10× dilutions on solid BG medium with the antibiotics corresponding to the mutant used.
8. Incubate at 28 °C for 48 h.
9. Count the bacterial colonies.

10. After 48–72 h in the growth chamber, depending of the experiment, sample again four discs of the other half of the same leaf with a punch borer and repeat **steps 5–9** (*see* **Note 13**).

3.7 Competitive Index Assays in Tomato Plants

1. Transfer the tomato plants in the growth chamber (28 °C day/27 °C night, 80–85% relative humidity, 12 h light) 2 days before the inoculation.
2. Prepare a small volume inoculum for each strain used for competitive index assay.
3. Mix 100 µL of a 10^8 CFU/mL suspension of strain A with 100 µL of a 10^8 CFU/mL suspension of strain B (Fig. 2) (*see* **Note 14**).
4. Serial-dilute the mixed inoculum to a final concentration of 10^6 CFU/mL and 10^4 CFU/ mL in 1 mL final volume.
5. Plate 100 µL of the 10^4 CFU/mL suspension on solid BG medium with the appropriate antibiotic for strain A and on solid BG medium with the appropriate antibiotic for strain B (Fig. 2) (*see* **Note 14**).

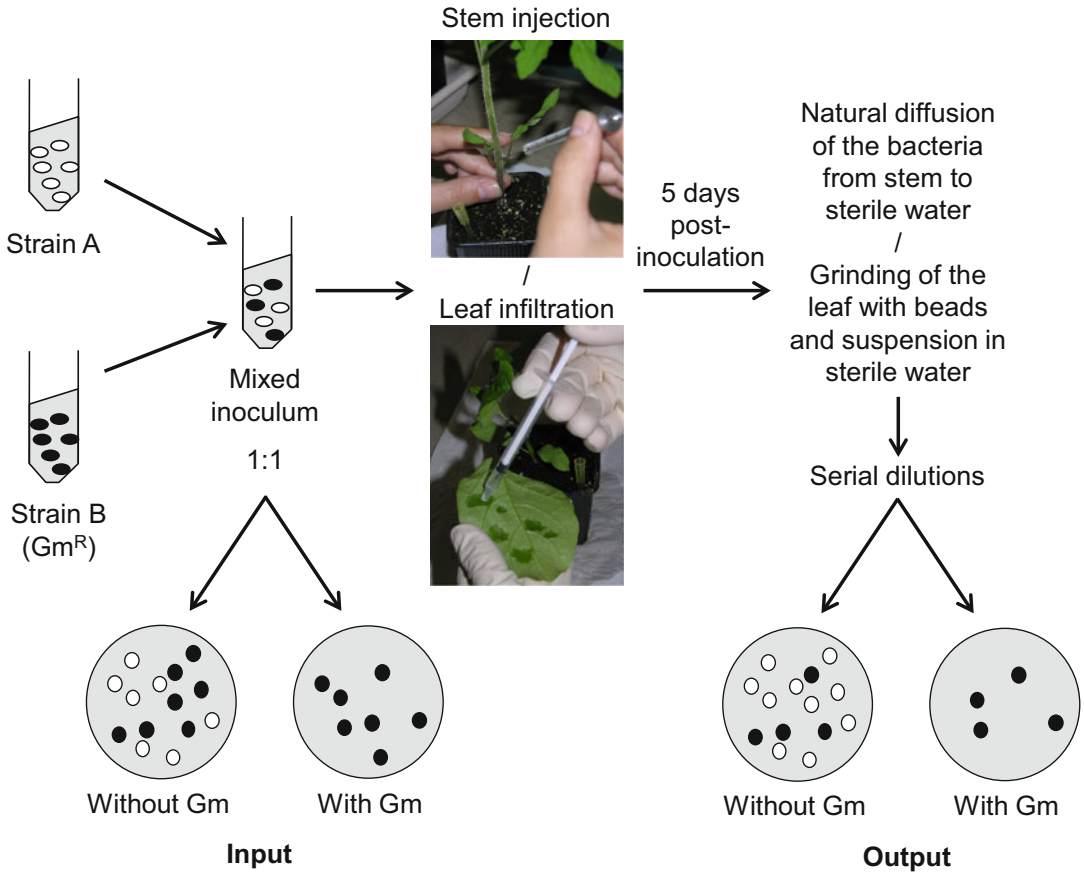

Fig. 2 Schematic representation of the competitive index assays (adapted from Macho et al. 2008 [18]). *Gm* gentamycin

6. Incubate at 28 °C for 48 h.
7. Count the bacterial colonies and calculate the ratio of strains A and B in the mixed inoculum which should be close to one (Input; Fig. 2).
8. For competitive index assays in tomato stem: inject 10 µL of the 10^6 CFU/mL mixed inoculum into the stem 0.5 cm above the cotyledons using a microsyringe. Between each mixed inoculum, sterilize the microsyringe by pumping up and down 70% ethanol three times and then sterile water three times. For competitive index assays in tomato leaf: infiltrate 50 µL of the 10^4 CFU/mL mixed inoculum into the leaf using a blunt syringe.
9. Incubate the plants in the growth chamber for 5 days.
10. After incubation, recover the bacteria in the inoculated plant material as follow (Fig. 2):

    For inoculated stems, sample the whole stem and petioles (without the leaves) 1 cm above the inoculation point. Sterilize the surface by putting the stem and petioles in 70% ethanol for 30 s, then rinse the stem and petioles by putting them in sterile water for 30 s. Truncate the stem and petioles into 1 cm segments with a sterile scalpel and put them in a Falcon tube containing 3 mL sterile water. Incubate at room temperature at least 30 min and less than 2 h so that the bacteria naturally diffuse from stem and petiole to sterile water.

    For inoculated leaves, sample four discs (7 mm diameter punch borer). Put the leaf discs in a 2 mL Eppendorf tube and grind them with a fixed amount of disposable 1 mm diameter glass beads at 30 Hz for 30 s with a mill-grinder. Place the tubes in a centrifuge and do a short spin to pellet all plant debris stuck on the Eppendorf lids. Add 1.8 mL sterile water and vortex.
11. Serial dilute the bacterial suspension and plate 100 µL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions on solid BG medium with the appropriate antibiotic for strain A and on solid BG medium with the appropriate antibiotic for strain B (Fig. 2) (*see* **Note 14**).
12. Incubate at 28 °C for 48 h.
13. Count the bacterial colonies and calculate the ratio of strains A and B in the Output (Fig. 2).
14. Calculate the competitive index (CI) as follow:

    CI = [Strain B CFU/Strain A CFU (output)]/[Strain B CFU/Strain A CFU (input)].
    CI = 1: B fitness = A fitness; CI < 1: B fitness < A fitness; CI > 1: B fitness > A fitness
15. Repeat at least five independent experiments to draw the conclusions.

16. Calculate the mean CI with SD and SE and perform a Wilcoxon test to conclude on differences between mean CI values [19].

3.8 Disease Development on Medicago truncatula Plantlets After R. solanacearum Root Inoculation Under In Vitro Conditions

1. Scarify the *Medicago* seeds with concentrated anhydrous H_2SO_4 (95%) for 7 min, and wash three times in sterile water.
2. Under a hood, sterilize the seeds for 2 min with a 12% sodium hypochlorite solution, and rinse six times with sterile water. Keep the seeds 30 min in the last water bath for an imbibition step.
3. Sow the seeds onto 0.8% (wt/vol) water agar in petri dishes (around 50 seeds per plate), seal it with Parafilm, and put the plates upside down for germination in the dark for 3 days at 4 °C, then 24 h at 14 °C.
4. Transfer germinating seeds (6–10 plants) on slanted agar in square petri dishes (12 × 12 cm) containing Fåhraeus medium and CYG seed growth paper. For this, transfer carefully the plantlets with curved extra fine tip tweezers, putting the hypocotyls at the upper paper/agar delimitation (Fig. 3). Seal the plates with Parafilm (on three sides, leaving the upper part without Parafilm), and incubate with an 45° angle for 3 days with the following conditions: 23 °C, 75% humidity, 16 h light at 100 μmol/m^2/s.
5. Inoculate 3-day-old plantlets in square petri dishes with 300 μL of a bacterial suspension at 10^7 CFU/mL brought to the tip of the root. Seal plates with Parafilm like previously.
6. Incubate the plants with a 45° angle in the growth chamber for 10–20 days with the following conditions: 28 °C the day/28 °C the night, 75% humidity, 12 h light at 100 μmol/m^2/s.

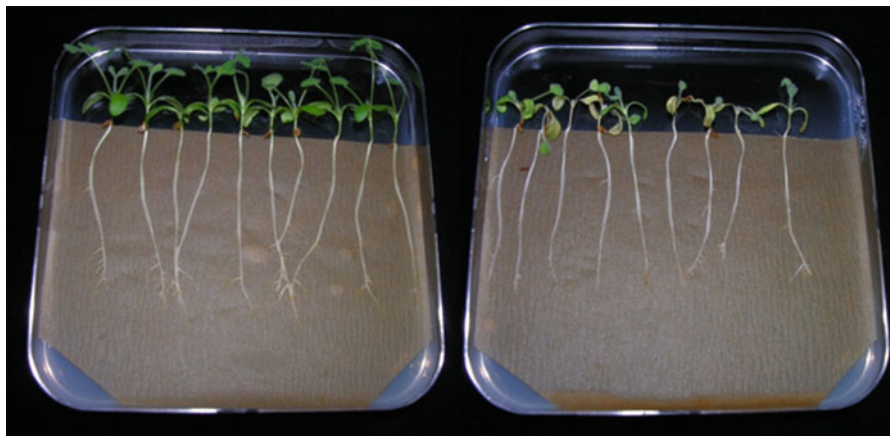

Fig. 3 Disease development on *Medicago truncatula* plantlets after *Ralstonia solanacearum* root inoculation under in vitro conditions. Inoculation with a virulent (right, chlorosis and wilting symptoms) or avirulent strain (left). Pictures taken 14 days after inoculation

7. Monitor disease development every day (chlorosis and wilting) from 10 to 20 days after inoculation (Fig. 3) (*see* **Note 6**).
8. Measure the bacterial multiplication in planta from 10 to 17 days after inoculation as follows: Surface sterilize at least three pools of three plantlets in a ethanol 70% bath for 1 min and rinse three times in sterile water baths for 1 min each. Per pool of three plantlets: weight, grind with a mortar and pestle and resuspend in 4 mL sterile water. Plate serial dilutions (10^{-1} to 10^{-5}) on solid BG medium with appropriate antibiotic. For statistical analyses, use a non parametric Mann Whitney test on series of biological repetitions to determine if the in planta bacterial multiplication of two strains can be considered different or not.

3.9 Inoculation of Arabidopsis Hydroponic Cultures

1. Surface sterilize *Arabidopsis* seeds. Mix 20 mg of seeds with 1 mL of 10% bleach solution in 95% ethanol solution in a 1.5 mL Eppendorf tube for 5–10 min, then wash twice with 95% ethanol and dry in the laminar flow hood for 1–2 h.
2. Prepare hydroponic culture units: cut the caps and 1 cm from the bottom of 0.5 mL Eppendorf tubes (Fig. 4a) and glue them on adhesive tape (Fig. 4a, b). Fill the culture units with half strength araponics solid medium diluted in water to a 1/4000 final concentration.
3. Transfer the hydroponic culture units in tip trays of a 1000 µL tip boxes filled with half strength araponics liquid medium diluted in water to a 1/4000 final concentration (*see* **Note 15**).
4. Sow 1–2 seeds in each hydroponic culture units. Close the tip box with transparent cover (Fig. 4c).
5. Grow *Arabidopsis* seedling for 10 days in a growth chamber with the following conditions: 22 °C, 70% relative humidity, 9 h light.
6. Transfer healthy seedlings (*see* **Note 16**) in pierced Plexiglas trays on 10 L bins filled with full strength araponics liquid medium diluted in water to a 1/2000 final concentration.
7. Grow *Arabidopsis* plantlets for 3 weeks in a growth chamber with the following conditions: 22 °C, 70% relative humidity, 9 h light. Change medium every 4 days.
8. Transfer the plants in the growth chamber for *R. solanacearum* inoculation with the following conditions: 27 °C the day/26 °C the night, 80–85% relative humidity, 12 h light. Replace araponics liquid medium by Fåhraeus liquid medium 7 h before inoculation.
9. Prepare a high volume inoculum to a final concentration of 10^8 CFU/mL in 10 L Fåhraeus liquid medium. The medium is aerated and the bacteria are kept in suspension using a 13 cm aquarium air pump with a ceramic diffuser (Fig. 4e).

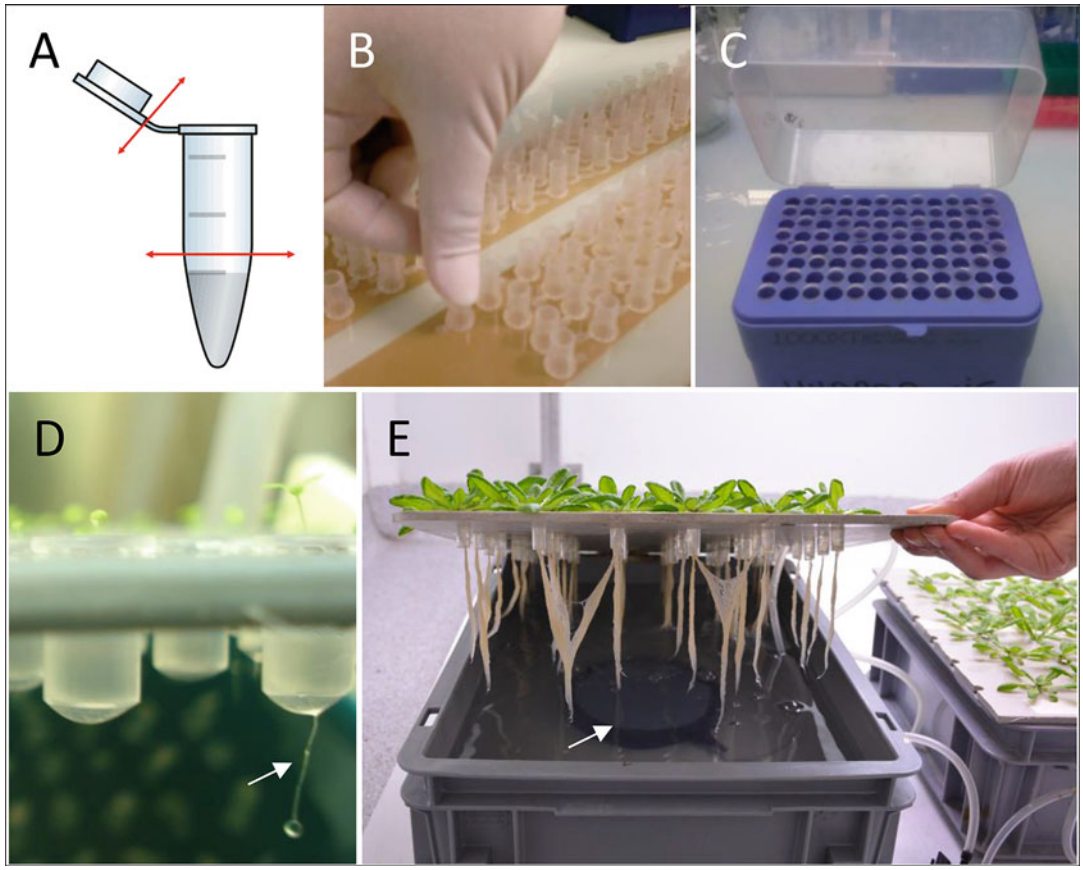

Fig. 4 *Arabidopsis* hydroponic culture setup and inoculation. 0.5 mL Eppendorf tubes are cut following red arrows (**a**) and glued on adhesive tape (**b**). Once filled with half strength araponics solid medium, hydroponic culture unit are displayed in trays of 1000 μL tip box filled with half strength araponics liquid medium (**c**). Healthy seedling for which root went through the solid medium (**d**; arrow) are transferred in pierced Plexiglas trays on 10 L bins filled with full strength araponics liquid medium (**e**). Inoculations are performed on 4-week-old *Arabidopsis* seedlings with a 10^8 CFU/mL bacterial suspension in Fåhraeus medium. Bacteria are kept in suspension using 13 cm aquarium air pump with a ceramic diffuser (e, arrow)

10. Incubate in the growth chamber for 10–20 days.
11. Once per day, assay wilting symptoms on a scale of 1–4 (Fig. 1) (*see* **Note 5**).

3.10 Measurement of Bacterial Multiplication in Roots Using a qPCR Method

1. Harvest the whole root system, wash the roots twice in two different sterile water baths for 1 min each, dry and transfer in a 2 mL Eppendorf tube containing a ceramic bead and 20 mg of sterile sand. Keep the samples frozen in liquid nitrogen until DNA extraction.
2. Generate two independent concentration ranges of bacteria (from 0 to 10^8 CFU/mL) by dilution from two independent overnight liquid cultures. Check the quantity of bacteria in each concentration range point by plating serial dilutions on

solid BG medium. Vacuum-infiltrate for 10 min 100 µL of each concentration range point in 100 mg fresh root tissue harvested from plant cultivated in hydroponic condition, in a 2 mL Eppendorf tube containing a ceramic bead and 20 mg of sterile sand. Centrifuge 10 min at 14,000 rpm in a tabletop centrifuge. Discard supernatant. Keep the samples frozen in liquid nitrogen until DNA extraction.

3. Grind the frozen samples with a Mill grinder (2 × 30 s at 30 Hz vibrational frequency) and extract the nucleic acids with TRIzol reagent using manufacturer's instructions. Perform an additional chloroform–isoamyl alcohol (24:1) extraction on 500 µL of aqueous phase. Add 1.25 mL absolute EtOH and 50 µL NaCl 5 M. Mix by inversion and incubate at −80 °C for 1 h. Precipitate nucleic acids by centrifugation at 13,000 rpm for 15 min at 4 °C in a refrigerated tabletop centrifuge. Discard supernatant and rinse pellet with 1 mL 75% Ethanol (diluted with RNase-free water). Vortex and centrifuge at 13,000 rpm for 30 min at 4 °C in a tabletop centrifuge. Discard supernatant, dry pellets at 65 °C for 2–5 min. Resuspend nucleic acids in 400 µL RNase-free water.
4. Measure DNA concentrations in black 96 well assay plates with clear flat bottom using quant-iT Picogreen dsDNA Assay Kit according to manufacturer's instruction. Adjust the DNA concentration of each sample to 4 ng/µL.
5. Perform qPCR in a 7 µL final volume containing 2 µL DNA, 36 pM final concentration of each primer, 3.5 µL LightCycler 480 SYBR Green 2× Master mix and 1.4 µL sterile water. Two primer pairs are used: EGL1ChF (5′-GCCGAAAGCA GACTACAACC-3′)/EGL1ChR (5′-TGGACAAATAGGGCT TGCT-3′) and AtEF1-F (5′-CTGGAGGTTTTGAGGCTG GTAT-3′)/AtEF1-R (5′-CCAAGGGTGAAAGCAAGAAGA-3′) (*see* **Note 17**). Perform qPCR in triplicate with the following conditions: 95 °C for 5 min; 40 × (95 °C for 10 s and 60 °C or 66 °C for 30 s for AtEF1-F/AtEF1-R or EGL1ChF/EGL1ChR primer pairs respectively) and a final dissociation step to perform melting curve analyses in order to assess the quality of qPCR products. The optimal cycle threshold (Ct) is determined by the LightCycler 480 Software version 1.5 provided by the manufacturer. Ct results obtained using EGL1ChF/EGL1ChR primers are normalized using results obtained with AtEF1-F/AtEF1-R primers.
6. Use the average qPCR Ct results from the two concentration ranges and the corresponding average bacterial quantity to establish a logarithmic regression curve. Use this curve to deduce the bacterial quantity in the root samples from the qPCR Ct results obtained from this root samples.
7. Repeat at least three independent experiments to draw the conclusions, each experiment containing 25 plants.

4 Notes

1. The 30 × 30 cm tray is not punctured for drainage and can thus contain the *R. solanacearum* inoculum.
2. It can be useful to dilute 100 µL of culture in 900 µL liquid BG medium to perform a accurate OD_{600nm} measurement with a spectrophotometer and plate the inoculum in order to have the exact bacterial concentration.
3. For some experiments, wounding of the root system is required. Cut the bottom third of the jiffy pots with scissors before soaking.
4. If assessing different strains, the minimal number of plants will depend on the difference in aggressivity among the reference and the tested strain. As a rule of thumb, when the relative aggressivity is not known or expected to be close, we perform each assay with a minimum of 20 plants. When different plant genotypes are assessed, we encourage randomizing the relative position of the different genotypes in order to do the visual scoring without knowledge of the relative position of the different plant genotypes.
5. The first symptoms should appear 3 or 4 days after inoculation for most experiments with optimal inoculation.
6. To compare the disease development of two given strains, the Kaplan–Meier survival analysis can be used with the Gehan–Breslow–Wilcoxon test done with the Prism version 5.00 (GraphPad Software) [11, 20, 21].
7. A higher concentration of 10^8 CFU/mL can be used if symptoms apparition seem delayed.
8. Be careful to correctly water the plants the day before inoculation as dry soil tends to be more difficult to soak leading to a nonhomogenous inoculation.
9. The bacterial load when assessed in the whole aerial part of the plant is well estimated by the bacterial load in this 1 cm stem section (unpublished data).
10. If wilting symptoms are observed, then plate the dilutions from 10^{-4} to 10^{-6}. If no wilting symptoms are observed, then plate the dilution 0 to 10^{-6} as important bacterial multiplication can occur before the first visual symptoms.
11. The wild type strain GMI1000 cannot be used for this experiment because *N. benthamiana* plants are resistant to this strain. The double mutant GRS473 (*popP1*::Ω *avrA*::Ω) has to be used [7].
12. Use the first big leaf starting from the top, as the older and younger leaves are sometimes difficult to infiltrate. If needed

poke a hole in the leaf with a needle to make the infiltration easier.

13. After 48 h, plate the dilutions 10^{-3} to 10^{-5}.
14. Competitive index assays are used to compare the fitness of two strains either into the stem or into the leaf of a plant. Usually, this approach is used to compare the fitness of a mutant and the corresponding wild-type strain. In order to differentiate the two strains, at least one of them must carry a specific marker such as an antibiotic resistance (gentamycin or spectinomycin resistance) or express of a fluorochrome (GFP or mCherry). When the two strains express different fluorochromes, it is possible to plate them on the same solid BG medium for the Input and the Output calculation (Fig. 2).
15. The bottom of the tubes must be soaked in the medium.
16. Healthy seedlings are seedlings with roots going through the solid medium and soaking in liquid medium (Fig. 4d).
17. The primer pair EGL1ChF/EGL1ChR, which amplifies a region of the unique endoglucanase gene (*EGL1*) localized on the *R. solanacearum* chromosome (EMBL AL646052), was designed in the present work with Primer3Plus online software. The primer pair AtEF1-F/AtEF1-R [9], which amplifies a region of the *AT5G60390* nuclear gene (GTP binding EFTu elongation factor), is used to normalize the results.

References

1. Mansfield J, Genin S, Magori S et al (2012) Top 10 plant pathogenic bacteria in molecular plant pathology. Mol Plant Pathol 13:614–629
2. Turner M, Jauneau A, Genin S et al (2009) Dissection of bacterial Wilt on *Medicago truncatula* revealed two type III secretion system effectors acting on root infection process and disease development. Plant Physiol 150:1713–1722
3. Vasse J, Genin S, Frey P et al (2000) The *hrpB* and *hrpG* regulatory genes of *Ralstonia solanacearum* are required for different stages of the tomato root infection process. Mol Plant-Microbe Interact 13:259–267
4. Peeters N, Guidot A, Vailleau F, Valls M (2013) *Ralstonia solanacearum*, a widespread bacterial plant pathogen in the post-genomic era. Mol Plant Pathol 14:651–662
5. Cunnac S, Occhialini A, Barberis P et al (2004) Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: identification of novel effector proteins translocated to plant host cells through the type III secretion system. Mol Microbiol 53:115–128
6. Poueymiro M, Genin S (2009) Secreted proteins from *Ralstonia solanacearum*: a hundred tricks to kill a plant. Curr Opin Microbiol 12:44–52
7. Poueymiro M, Cunnac S, Barberis P et al (2009) Two type III secretion system effectors from *Ralstonia solanacearum* GMI1000 determine host-range specificity on tobacco. Mol Plant-Microbe Interact 22:538–550
8. Lavie M, Shillington E, Eguiluz C et al (2002) PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. Mol Plant-Microbe Interact 15:1058–1068
9. Le Roux C, Huet G, Jauneau A et al (2015) A receptor pair with an integrated decoy converts pathogen disabling of transcription factors to immunity. Cell 161:1074–1088
10. Angot A, Peeters N, Lechner E et al (2006) *Ralstonia solanacearum* requires F-box-like domain-containing type III effectors to promote disease on several host plants. Proc Natl Acad Sci U S A 103:14620–14625

11. Wang K, Remigi P, Anisimova M et al (2016) Functional assignment to positively selected sites in the core type III effector RipG7 from *Ralstonia solanacearum*. Mol Plant Pathol 17:553–564
12. Chen L, Shirota M, Zhang Y et al (2014) Involvement of HLK effectors in *Ralstonia solanacearum* disease development in tomato. J Gen Plant Pathol 80:79–84
13. Solé M, Popa C, Mith O et al (2012) The *awr* gene family encodes a novel class of *Ralstonia solanacearum* type III effectors displaying virulence and avirulence activities. Mol Plant-Microbe Interact 25:941–953
14. Chen Y, Zhang W-Z, Liu X et al (2010) A real-time PCR assay for the quantitative detection of *Ralstonia solanacearum* in the horticultural soil and plant tissues. J Microbiol Biotechnol 20:193–201
15. Macho AP, Guidot A, Barberis P et al (2010) A competitive index assay identifies several *Ralstonia solanacearum* type III effector mutant strains with reduced fitness in host plants. Mol Plant-Microbe Interact 23:1197–1205
16. Fåhraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. Microbiology 16:374–381
17. Vailleau F, Sartorel E, Jardinaud M-F et al (2007) Characterization of the interaction between the bacterial wilt pathogen *Ralstonia solanacearum* and the model legume plant Medicago truncatula. Mol Plant-Microbe Interact 20:159–167
18. Macho AP, Zumaquero A, Ortiz-Martín I, Beuzón CR (2008) Competitive index in mixed infection: a sensitive and accurate method to quantify growth of pseudomonas syringae in different plants. In: Fatmi MB et al (eds) Pseudomonas syringae pathovars and related pathogens – identification, epidemiology and genomics. Springer, Dordrecht, pp 191–203
19. Guidot A, Jiang W, Ferdy J-B et al (2014) Multihost experimental evolution of the pathogen *ralstonia solanacearum* unveils genes involved in adaptation to plants. Mol Biol Evol 31:2913–2928
20. Remigi P, Anisimova M, Guidot A et al (2011) Functional diversification of the GALA type III effector family contributes to *Ralstonia solanacearum* adaptation on different plant hosts. New Phytol 192:976–987
21. Lonjon F, Turner M, Henry C et al (2016) Comparative secretome analysis of *ralstonia solanacearum* type 3 secretion-associated mutants reveals a fine control of effector delivery, essential for bacterial pathogenicity. Mol Cell Proteomics 15:598–613