

An Innovative Approach to Study *Ralstonia solanacearum* Pathogenicity in 6 to 7 Days Old Tomato Seedlings by Root Dip Inoculation

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[Abstract] *Ralstonia solanacearum* (F1C1) is a Gram-negative plant pathogenic bacterium that causes lethal wilt disease in a wide range of plant species. This pathogen is very well known for its unpredictable behavior during infection and wilting its host. Because of its mysterious infection behavior, virulence and pathogenicity standardization are still a big challenge in the case of *R. solanacearum*. Here, we report an innovative pathogenicity assay of *R. solanacearum* (F1C1) in the early stage of tomato seedlings by root dip inoculation. In this assay, we employed 6-7 days old tomato seedlings for infection grown under nutrients free and gnotobiotic condition. After that, pathogenicity assay was performed by maintaining the inoculated seedlings in 1.5 or 2 ml sterile microfuge tubes. During infection, wilting symptom starts appearing from ~48 h post inoculation and the pathogenicity assay gets completed within seven days of post inoculation. This method is rapid, consistent as well as less resource dependent in terms of labor, space and cost to screen large numbers of plants. Hence, this newly developed assay is an easy and useful approach to study pathogen virulence functions and its interaction with the host plant during wilting and disease progression at the seedling stage.

Keywords: *Ralstonia solanacearum*, Root inoculation, Tomato seedling, Pathogenicity, Wilting

[Background] Bacterial wilt pathogen *Ralstonia solanacearum* dwells in soil. When favorable condition comes, the bacterium enters inside the suitable host plant through root, grows, colonizes there and ultimately kills the plant. *R. solanacearum* strain (F1C1) had been isolated and characterized from a wilted chilli plant nearby Tezpur (Assam), north-east India (Kumar *et al.*, 2013). Owing to the lethality and its exceptional wide host range, *R. solanacearum* considered as the second most devastating bacterial phytopathogen around the world (Mansfield *et al.*, 2012). In spite of that, no effective approach so far is available to deal with this pathogen and its associated disease.

Regarding its virulence and pathogenicity assay, several existing methods are commonly in use such as soil drenching, leaf clip, petiole cut as well as stem inoculation but, in the other hand it is also reported that these methods are not found to be very much appropriate in analyzing minute virulence and pathogenicity differences in few mutants strain of *R. solanacearum* (Macho *et al.*, 2010). In the wake of developing a potent pathogenicity assay, we have developed and standardized an innovative root inoculation method to study *R. solanacearum* pathogenicity in tomato seedling (Singh *et al.*, 2018). Along with the pathogenicity assay (Figure 1), this approach is also equally helpful in bacterial bio-

control development and plant protection assay against this wilt pathogen at the seedling stage. Recently many other research groups also have employed seedling stages of tomato plants for studying *R. solanacearum* pathogenicity in number of occasions (Pradhanang *et al.*, 2000; Artal *et al.*, 2012; Kumar, 2014; Kumar *et al.*, 2017). This vascular pathogen is well known for its mysterious infection behavior and wide range of host adaptability, therefore *R. solanacearum* is also referred as a suitable model to investigate fundamental aspects of plant-pathogen interaction and host adaptations (Genin and Boucher, 2002; Genin, 2010; CollandValls, 2013; Singh *et al.*, 2018).

Materials and Reagents

1. 1.5 ml and 2.0 ml micro centrifuge tubes (Tarson, catalog numbers: 500010 and 500020)
2. Pipette tips (10 µl) (Tarson, catalog number: 521000)
3. Pipette tips (200 µl) (Tarson, catalog number: 521010)
4. Pipette tips (1,000 µl) (Tarson, catalog number: 521020)
5. 13 mm screw vial (Borosil, catalog number: VO04C113005000)
6. 30 ml culture tubes (Riviera, catalog number: 71200305)
7. 50 ml Falcon tube (Tarson, catalog number: 546041)
8. 250 ml conical flask (Borosil, catalog number: 4980021)
9. Plastic tray (13 cm x 22 cm)
10. Petri dishes (90 mm) (Tarson, catalog number: 460090)
11. 120-well 1.5 ml to 2.0 ml tube rack (Tarson)
12. Meta loop (Himedia, catalog number: LA650-1)
13. Cotton wool(SN surgical & health care science (P) LTD, catalog number: DL-160M)
14. Tissue paper (Hygienics Plus, catalog number: HPTP02)
15. *Ralstonia solanacearum* (F1C1) (Lab collection)
16. Tomato seeds (Durga: Selection-22) [Durga seed farm(REGD), Chandigarh, India]
17. Sterile distilled water
18. Peptone (Himedia, catalog number: RM001-500G)
19. Casamino acid or casein acid hydrolysate (SRL, catalog number: CI019)
20. Glucose (Himedia, catalog number: MB037-500G)
21. Agar powder (Himedia, catalog number: GMR026-500G)
22. Yeast extract powder (Himedia, catalog number: RM027-500G)
23. BG agar media (see Recipes)
24. BG broth media (see Recipes)
25. 20% Glucose supplement (see Recipes)

Equipment

1. Pipettes (Eppendorf-these pipettes can accommodate pipette tips of 10 µl, 200 µl, 1,000 µl respectively)
2. Water distillation system (Riveria, catalog number: 7222010)
3. Autoclave (EquitronMedica, model: 7431PAD)
4. Incubator and shaker (28 °C) (Scigenics Biotech, Orbitek®, model: LE0102DCBA)
5. Macro centrifuge (Eppendorf, model: 5804R)
6. Laminar Air Flow (Ikon Instruments, model: 1K-137)
7. Weighing balance (Mettler Toledo, model: ME204)
8. Growth chamber (ScigenicsBiotech, Orbitech®, model: GC350)

Procedure

A. Germination of tomato seedling (Figure 1)

1. Take tomato seeds and wash them with sterile distilled water.
2. Soak seeds in sterile distilled water for 48 h at room temperature of ~25 to 30 °C (Figure 1A).
3. Spread soaked seeds on cotton and tissue bed in a plastic or glass tray, and then put the tray for germination in a growth chamber maintained at 28 °C, 75% RH and 12 h of photoperiod (Light intensity of the lamp as 1,350 lm and the color temperature as 4,000 K) (Figure 1B).
4. Maintain the moisture of the seed bed by spraying sterile distilled water at regular interval during the seed germination.

Note: The spray of sterile distilled water at regular interval help in better seedling growth and it also helps to prevent bacterial and/or fungal contamination.

5. Pick the 6-7 days old tomato seedlings with similar size for infection assay.

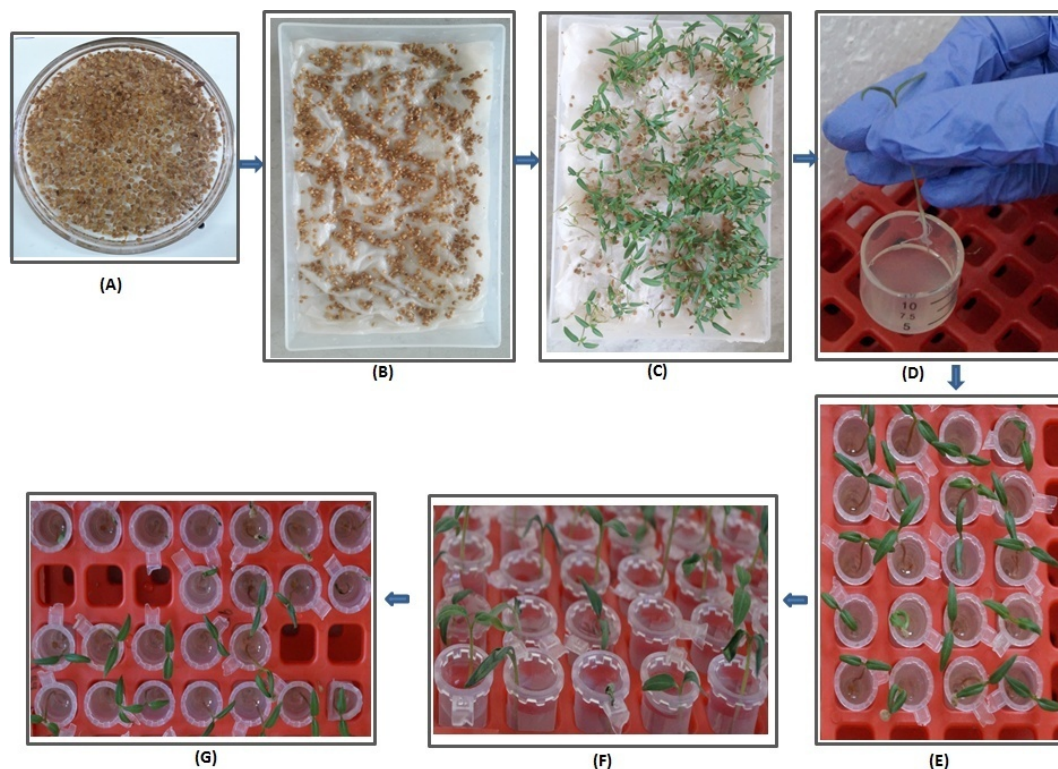


Figure 1. Pictures showing the different steps of root inoculation assay to study *R. solanacearum* pathogenicity in tomato seedlings. A. Soaking of tomato seeds in sterile distilled water for 48 h. B. Spreading of seeds on sterile and wet cotton and tissue paper bed. C. Germination of tomato seeds on a sterile and wet tissue paper bed in a growth chamber at 28 °C and 75% relative humidity up to the seedling stage. D. Root inoculation of tomato seedling by dipping the root in bacterium inoculums container. E. Each inoculated seedling transferred to sterile 1.5-2 ml empty microfuge tubes. After approximately 5 min of air exposure, 1.0 to 1.5 ml of sterile water was added to each microfuge tube. F. Approximately 40-48 h post inoculation, infected seedlings started wilting indicating the early wilting symptom. G. On the 7th day post inoculation, 80% to 90% of infected tomato seedlings became permanently wilted and died (top side) in comparison to water as control (bottom side).

B. Bacterial inoculum preparation

1. Streak *R. solanacearum* (F1C1) bacterium on BG-Agar plate and incubate in an incubator (Orbitek, Scigenics Biotech (Pvt.) Ltd., India) maintained at 28 °C for 24 h.
2. Add freshly grown *R. solanacearum* (F1C1) colonies to 50 ml BG broth in a sterile vial with a sterile loop and allow to grow in a shaking incubator (Orbitek, Scigenics Biotech (Pvt.) Ltd., India) maintained at 28 °C and 150 rpm for 24 h.
3. Centrifuge the full-grown bacterial cultures at 4,000 rpm (3,155 x g) for 15 min at 4 °C and then discard the supernatant.
4. Resuspend bacterial pellets in sterile distilled water and repeat Step B3 (centrifuge at 4,000 rpm [3,155 x g] for 15 min at 4 °C) to remove remaining culture media component from the

suspension.

5. Now resuspend bacterial pellet in an equal volume of sterile distilled water to obtain a concentration of $\sim 10^9$ CFU/ml. Now, this bacterial inoculum is ready for infection.

C. Inoculation of *R. solanacearum* in 6-7 days old tomato seedlings (Figure 2)

1. Pick 6-7 days old tomato seedlings with similar size from germination bed.
2. Add 5-20 ml of bacterial inoculum into a wide mouth container.
3. Immerse the seedling roots in bacterial inoculum up to the root-shoot junction for a few seconds.
4. Now transfer these seedlings to an empty and sterile microfuge (1.5 or 2.0 ml) tube.
5. Then air expose the seedlings for ~ 5 min (Here “air expose” means that pathogen inoculated seedlings are placed in a sterile empty microfuge tube for ~ 5 min before addition of water to the tube).
6. Add 1 to 1.5 ml of sterile distilled water to the microfuge tube containing the air exposed seedlings.
7. Inoculate seedlings in the same way with sterile distilled water as a negative control.
8. Transfer all the inoculated as well as control seedlings to a growth chamber maintained at 28 °C, 75% RH, and 12 h of photoperiod.
9. Start infection and disease progression analysis from the next day onwards till the 7th day post inoculation.

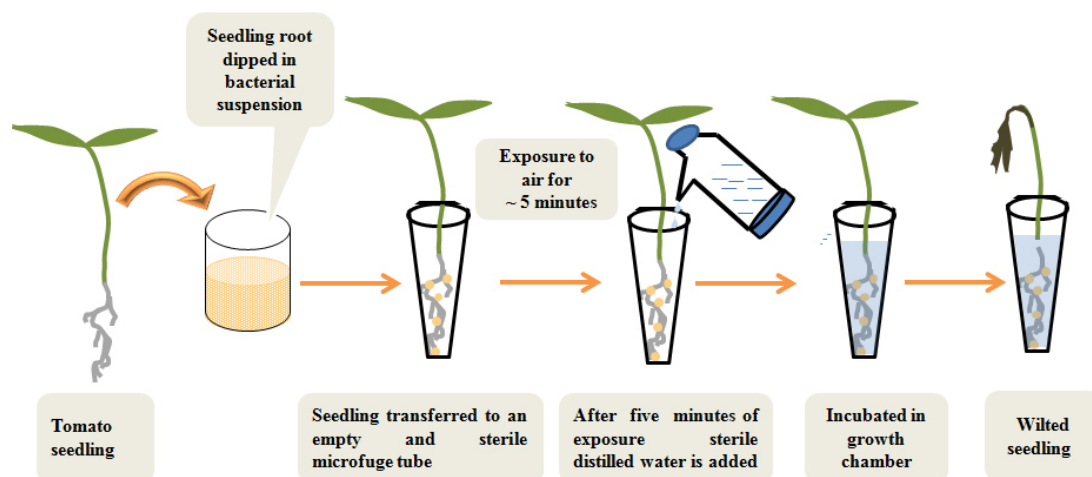


Figure 2. Schematic diagram of root inoculation of tomato seedlings in the *R. solanacearum* pathogenicity assay. Here, we are representing self-explanatory schematic diagram of pathogenicity assay for easy and better understanding of the crucial steps of the assay.

Data analysis

In this work, we have successfully described details of an easy and efficient approach to study *R. solanacearum* pathogenicity in the early seedlings stage of tomato cultivars. The described assay is very much reproducible and appropriate to analyze the different kind of mutant gene function including minute virulence function in *R. solanacearum*.

In addition to above, during infection assay, we have observed and first time reported an interesting air influence (Air exposed pathogen inoculated tomato seedling shown more aggressive and consistent disease progression in comparison to unexposed one) phenomenon on *R. solanacearum* pathogenicity in tomato seedlings by root inoculation. After bacterial inoculation, tomato seedlings will appear wilting symptom within 48 h and became completely wilted within 7 days post inoculation. This inoculation method mimics natural mode of infection caused by this wilt pathogen. Considering the reproducibility and efficiency of this root inoculation method in early seedling stage of tomato, it would help the concerned scientific community to study the virulence behavior, host adaptability as well as many other fundamental aspects of plant-microbe interaction during *R. solanacearum* infection and disease progression.

All the other required information about data processing, statistical analysis as well as details of replicates and independent experiments were already included in original research paper Singh *et al.* (2018).

Notes

1. As tomato seeds germination are not 100% efficient, this should be taken into consideration while designing the experiment.
2. Add some amount of water to the germination bed just before starting the tomato seedling picking for root inoculation which will help in smooth picking as well as minimizing the injury or breakage in root seedling.
3. Although we have maintained ~5 min of air exposure to get better infection result but, postinoculation air exposure for a fraction of second is also enough to induce disease symptom.
4. This assay was validated with the help of non-pathogenic bacterium strain inoculation as well as different mutant derivative strains of *R. solanacearum* (F1C1) such as *hrpB*, *phc A* and *pilT*. After that, further bacterial colonization studies inside inoculated and wilted seedlings were also confirmed by GUS staining and mCherry-fluorescence (Singh *et al.*, 2018).
5. All these bacterial inoculation and infection steps were performed on clean and surface sterilized workbench inside the laboratory. For infection and pathogenicity assay steps sterile hood with laminar flow would not be required.

Recipes

1. BG-Agar, pH 6.5-7 (100 ml)
 - 1 g Peptone
 - 0.1 g Casamino acid
 - 0.1 g Yeast extract
 - 1.5 g Agar

Add distilled water up to 100 ml and autoclave the media at 121 °C (249 °F) for around 15-20 min

Supplement 500 µl of 20% glucose to it and pour into sterile Petri plates, let the plates completely solidify
2. BG-Broth, pH 6.5-7 (100 ml)
 - 1 g Peptone
 - 0.1 g Casamino acid
 - 0.1 g Yeast extract

Add distilled water up to 100 ml and autoclave the media at 121 °C (249 °F) for around 15-20 min. Supplement 500 µl of 20% glucose to it
3. 20% Glucose supplement (5 ml)
 - 1 g glucose

Add distilled water up to 5 ml and autoclave it at 121 °C (249 °F) for around 15-20 min

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Competing interests

The authors have declared no conflict of interest.

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