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A Quick Method for Screening Biocontrol Efficacy of Bacterial Isolates Against Bacterial Wilt Pathogen *Ralstonia solanacearum* in Tomato

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[Abstract] *Ralstonia solanacearum* is a bacterial phytopathogen able to cause bacterial wilt disease in more than 200 plant species. Plant disease biocontrol strategies are used for controlling this disease and tomato is used as a model plant to conduct *R. solanacearum* associated studies. Conventional screening methods such as seed bacterization, soil drenching and root bacterization (in grown plants) to assess the ability of biocontrol bacteria to antagonize *R. solanacearum* under *in planta* conditions in different hosts are time-consuming and costly. A fast, cost effective method is a key requirement to advance the research on *R. solanacearum* biocontrol. In this protocol, we have inoculated the roots of tomato seedlings with bacterial isolates showing antagonistic activity against *R. solanacearum* under *in vitro* conditions. After 16 h of treatment with the antagonizing bacteria, seedlings were inoculated with *R. solanacearum* by a well-established root-dip method. Then the seedlings were maintained at controlled conditions and the number of wilted/dead seedlings were recorded up to 10th day post *R. solanacearum* inoculation. Biocontrol efficacy was calculated from the records for each tested isolate. This protocol is advantageous than already available protocols in the sense that it can be completed within a very short duration (~18 days for tomato) and there is no requirement of culture media to maintain the seedlings. This method can be used for quickly screening large number of bacterial isolates and different host genotypes within a short period of time and at a minimum cost.

Keywords: Bacterial wilt, *Ralstonia solanacearum*, Biocontrol agent, Biocontrol efficacy, Tomato

[Background] *Ralstonia solanacearum* is a soil-borne bacterial phytopathogen causing bacterial wilt disease in more than 200 crop species representing 50 different families (Seleim *et al.*, 2014), the majority from the family Solanaceae (Thera *et al.*, 2010). It can thrive up to several years in the soil retaining the capability to infect upon arrival of a host (Coutinho and Wingfield, 2017). Its natural route of entry is through the roots and finally, it ends up colonizing in the xylem vessels causing blockage of water conduit due to secreted exopolysaccharides with a corresponding wilting symptom observed in the leaves (Genin and Denny, 2012; Peeters *et al.*, 2013; Seleim *et al.*, 2014). Despite having usual practices of disease control such as the use of resistant plant varieties, industrial pesticides (Yuliar *et al.*, 2015), the use of biocontrol agents is a much-preferred choice in the current scenario due to environment friendly nature of the biocontrol agents. Bacteria and bacteriophages have been reported as biocontrol agents against *R. solanacearum*, of which bacteria being the predominant (Yuliar *et al.*, 2015). The assay for the potential to antagonize *R. solanacearum* under *in vitro* conditions is quite easy

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as standard methods like agar well diffusion (Balouiri *et al*., 2016), disc diffusion (Gupta *et al*., 2015) and spot-on lawn assay (Vijayakumar and Muriana, 2015) are available. To be considered as a biocontrol agent of practical use it must primarily have the ability to control the pathogen under *in planta* condition and it should not have any negative effect on other living beings. The ability of self-sustainability and neutral effect on other living beings is a subject of later consideration, the first and foremost being its ability to antagonize the pathogen under *in planta* condition (Wang *et al*., 2019). Conventional methods of bacterial biocontrol agent screening against *R. solanacearum* under *in planta* conditions such as seed bacterization (Siddiqui and Meon, 2009) and soil drenching (Van Elsas and Heijnen, 1990) have several limitations as they require viz. 1) large amount of inoculum, 2) prolonged maintenance of plants and 3) large space. In general, these demerits sum up these methods as time consuming and costly. To further advance the research on biocontrol bacteria against *R. solanacearum*, a quick screening method escaping the limitations of above-mentioned methods is required. Considering the entry of this pathogen through the roots, it was hypothesized that the application of biocontrol bacteria in the root would have the maximum chance to antagonize *R. solanacearum* thereby attenuating or eliminating its disease-causing capacity. The method described here is easy to perform and economically viable as it requires very less resource and time. The method is useful to screen biocontrol efficacy of several bacterial isolates simultaneously against *R. solanacearum* in a very limited space.

Materials and Reagents

1. Micro centrifuge tube 1.5 ml (Tarsons, catalog number: 500010)
2. Petri dish 90 mm (Tarson, catalog number: 460050)
3. Petri dish 60 mm (Tarson, catalog number: 460061)
4. Microtips 200-1,000 µl (Tarson, catalog number: 521020)
5. Microtips 2-200 µl (Tarson, catalog number: 521010)
6. Microtips 0.2-10 µl (Tarson, catalog number: 521000)
7. Absorbent cotton wool (Bengal Surgicals Limited, India/Azpack, catalog number: 12356477)
8. Tissue paper roll (Solimo/Kimberly-Clark WypAll L10 Paper Wipes, catalog number: 13478218)
9. Polygrid micro tube stand (Tarson, catalog number: 205110)
10. Plastic tray (Agrawal Plastic and Packaging Industries)
11. Cotton plugs (Prepared in laboratory)
12. Spray bottle (Abdos, catalog number: P11191)
13. Inoculating loop (Himedia, catalog number: LA014)
14. Hand gloves (Kimtech, catalog number: 97613)
15. Spatula (Himedia, catalog number: LA007)
16. Parafilm (Himedia, catalog number: LA017)
17. HiDispo™ Bag-12 (Himedia, catalog number: PW040)
18. Tomato seeds var. Pusa Ruby (JAI Kisan Seeds Private Limited, India)

19. *R. solanacearum* antagonizing bacteria (*Staphylococcus warneri* GL1, *Bacillus velezensis* GL3, *B. velezensis* GL5 and *B. velezensis* GMC2) isolated in authors laboratory from the endosphere of *Gnetum gnemon*
20. *R. solanacearum* F1C1 strain (provided by Prof. S.K. Ray, Tezpur university)
21. 70% ethanol (Himedia, catalog number: MB106-500ML)
22. 1% NaOCl (Himedia, catalog number: AS102-100ML)
23. Sterile distilled water
24. Peptone (Himedia, catalog number: RM001-500G)
25. Acicase (Himedia, catalog number: CR013-500G)
26. Glucose (Himedia, catalog number: MB037-500G)
27. Nutrient broth (Himedia, catalog number: M002-500G)
28. Glycerol (Himedia, catalog number: AS100-500ML)
29. Agar powder (Himedia, catalog number: CR301-500G)
30. Casamino acid peptone glucose broth (see Recipes)

Equipment

1. Beaker (Borosil, catalog number: BRL_1060D21)
2. Erlenmeyer flask 250 ml (Borosil, catalog number: 4980021)
3. Glass beaker (Borosil, catalog number: 1000D121)
4. Forceps (Tarsons, catalog number: 486000)
5. Spirit lamp/alcohol burner lamp (Himedia, catalog number: LA275)
6. Growth Chamber (Hipoint, model: 740FHLED)
7. Orbital shaking incubator (Remi, model: CIS-24 plus)
8. Refrigerated centrifuge (Sigma model: 2-16KL)
9. Autoclave (Optics, model: 50 litres)
10. Water purification system (Sartorius, model: Arium mini Plus UV)
11. Pipettor (Eppendorf Research Plus, catalog numbers: 3120000020, 3120000046, 3120000062)
12. Weighing balance (Shimadzu, model: BL220H)
13. Laminar air flow cabin (Cleanair Systems, model: CAH 1200)
14. Eppendorf Biophotometer (Eppendorf, model: D30)

Software

1. SPSS 20.0
2. Microsoft Office Excel 2016

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Procedure

A. Tomato seedling preparation

1. Surface sterilize the tomato seeds by treating with 70% ethanol for 5 min followed by 1% NaOCl for 5 min and five washes with sterile water under laminar air flow cabin.
2. Take the seeds in a glass beaker with sterile water, stir gently and wait for 10 min.
3. Discard the seeds floating on surface of water after 10 min to maximize the number of potentially viable seeds.
4. Keep the beaker with the remaining seeds at 28 °C, in the dark for 24 h.
5. Wash the soaked seeds three times with sterile water.
6. Prepare a three-layered germination bed–sterile tissue paper on top, 3 cm thick sterile cotton in the middle and a polythene layering (HiDispoTM Bag) at the bottom. Place this bed in a plastic tray (30 × 20 cm^2) and soak the bed with sterile water (Video 1).

Video 1. How to prepare germination bed

7. Spread the seeds uniformly on the bed and place this germination tray in a growth chamber conditioned at 28 °C, 75% relative humidity and 12 h of photoperiod (light intensity 450 µmol m^{-2} s^{-1}).
8. Spray sterile water on a regular basis to sustain the growth of seedlings.
9. Five days old seedlings are used for priming with *R. solanacearum* antagonizing bacterium.

B. Antagonistic bacterial inoculum preparation

1. Culture the *R. solanacearum* antagonizing bacterium individually in nutrient broth medium at 28 ± 2 °C in shaking condition (120 rpm) for 24 h in the dark.
2. Centrifuge the culture broth at 430 *x g* for 10 min at room temperature and discard the supernatant. Resuspend the cell pellet in sterile water without vortexing.
3. Repeat the above step two more times and adjust the cell density of final suspension to ~10^8

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CFU ml^{-1} (OD = 0.4). About 10 ml *R. solanacearum* antagonizing bacterial cell suspension is sufficient to prime 30 seedlings.

C. Seedling root bacterization with antagonistic bacterium

1. Uproot 5 days old tomato seedlings from the germination tray without damaging the root system.
2. Put 30 seedlings in a Petri dish (60 mm diameter, 10 mm height) with 10 ml of *R. solanacearum* antagonizing bacterial cell suspension. Ensure the contact of the root with the bacterial suspension (Figure 1).

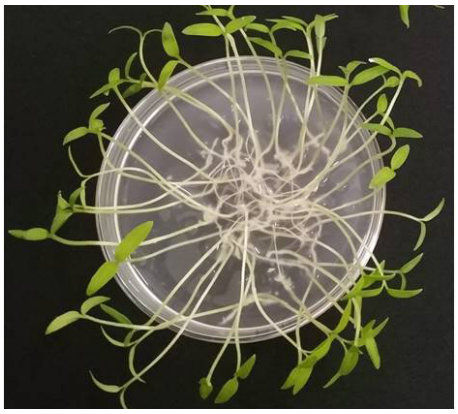

Figure 1. Seedling root bacterization

3. Keep the Petri dish with seedlings in a growth chamber conditioned at 28 °C, 75% relative humidity, and 12 h of photoperiod for 16 h (light intensity 450 µmol m^{-2} s^{-1}).
4. Treat 30 seedlings with sterile water to serve as a negative control group, which will be inoculated later with *R. solanacearum* (CK+R in Table 1).
5. Mock inoculate another 30 seedlings with sterile water to act as control group to nullify the natural death of seedlings in hydroponic conditions without being exposed to any bacteria (CK+W in Table 1).
6. After 16 h of priming with antagonizing bacteria, seedlings are ready for *R. solanacearum* inoculation.

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Table 1. The types of treatment groups required in this method with description and purpose

Group	Treatment	Purpose
Control group-1 (CK+W)	Neither inoculation with antagonizing bacterium nor *R. solanacearum*	To nullify the effect of naturally wilted/died seedlings in the treatment groups. Seedlings should not wilt/die in this group.
Control group-2 (CK+R)	Mock inoculation with sterile water followed by *R. solanacearum* inoculation	To act as a negative control group. All or most of the seedlings should wilt/die.
Test group (GL1+R, GL3+R, GL5+R, GMC2+R)	Inoculation with antagonizing bacterium followed by *R. solanacearum* inoculation	To calculate biocontrol efficacy of antagonizing bacterium. Number of wilted/died seedlings will depend on the biocontrol efficacy of the antagonizing bacterium being tested.

D. *R. solanacearum* inoculum preparation

1. Culture *R. solanacearum* in casamino acid-peptone-glucose (CPG) broth at 28 ± 2 °C in shaking condition (120 rpm) for 24 h in the dark.
2. Centrifuge the culture broth at 430 *x g* for 10 min at room temperature and discard the supernatant. Resuspend the cell pellet in sterile water without vortexing.
3. Repeat the above step two more times and adjust the cell density of final suspension to ~10^8 CFU ml^{-1}.

E. Inoculation of *R. solanacearum* in antagonistic bacteria primed seedlings

1. Take out the seedlings from the Petri dish and keep them in another sterile, dry Petri dish before *R. solanacearum* inoculation. Use these seedlings for inoculation immediately within 5 min, otherwise seedlings may dry out.
2. Inoculate the seedlings with *R. solanacearum* by root-dip inoculation method described by Singh *et al.* (2018). Briefly, take the *R. solanacearum* suspension in a wide mouth tube and immerse the seedling in the inoculum upto the root shoot junction for a few seconds.
3. Seedlings of the group CK+W should not be inoculated with *R. solanacearum*. It is kept as control.
4. Place the inoculated seedlings in 1.5 ml microcentrifuge tube and then add 1 ml sterile water into it. Use one seedling per microcentrifuge tube.
5. Place the microcentrifuge tube in a rack and keep inside a growth chamber conditioned at 28 °C, 75% relative humidity, and 12 h of photoperiod for 10 days (light intensity 450 µmol m^{-2} s^{-1}). Add an equal amount of sterile water to the tubes on a regular basis to sustain the viability of seedlings.
6. A seedling is considered wilted when the leaves turn yellow to dark brown and the shoot bends at a sharp angle along the side of the microcentrifuge tube (Figure 2).

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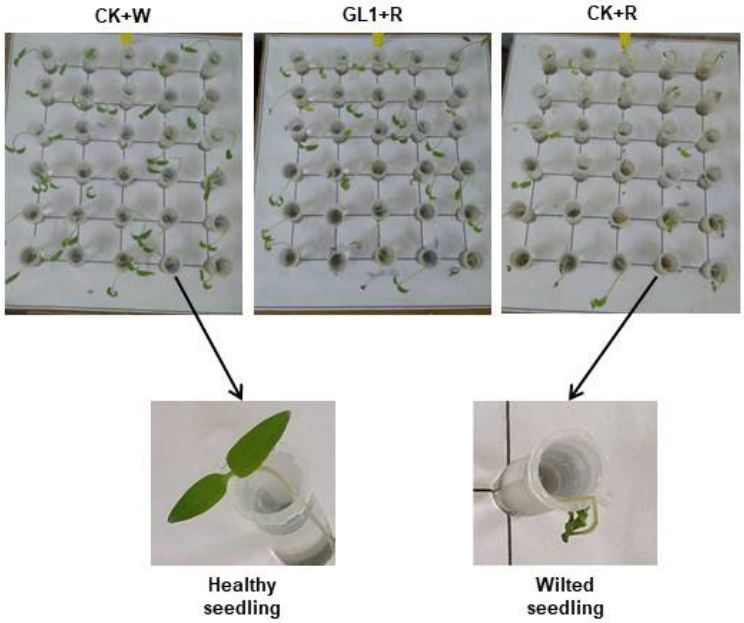

Figure 2. Effect of different treatment on tomato seedlings after 7 days of *R. solanacearum* inoculation. CK+W: neither inoculation with antagonizing bacterium nor *R. solanacearum,* GL1+R: inoculation with antagonizing bacterium *Staphylococcus warneri* GL1 followed by *R. solanacearum* inoculation, and CK+R: mock inoculation with sterile water followed by *R. solanacearum* inoculation. This figure is reprinted (with slight modification) from original research article Agarwal *et al.* (2020) with permission from Elsevier.

7. Keep records of wilted seedlings on a daily basis from 1st day to 10th day post *R. solanacearum* inoculation at an interval of 24 h.
8. A stepwise demonstration of the protocol is given in Figure 3.

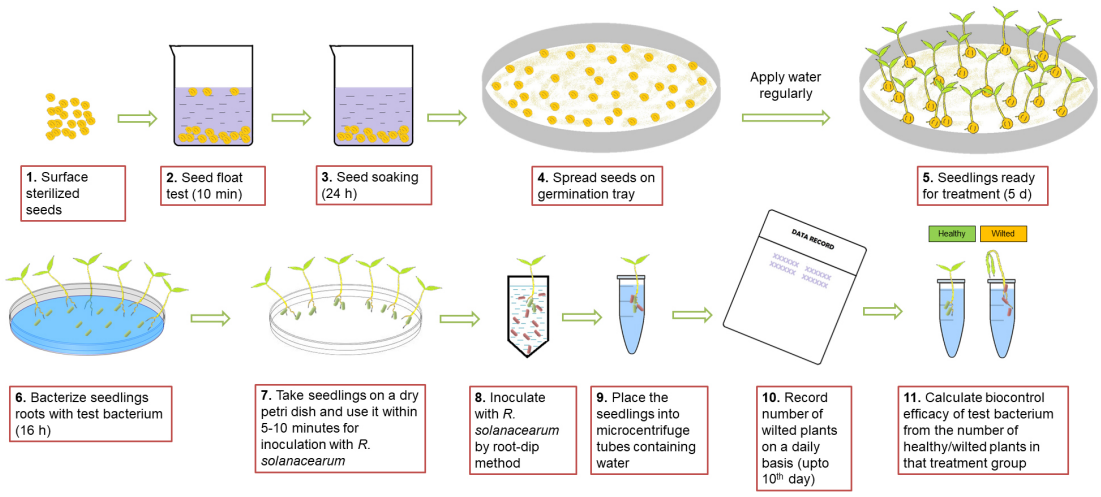

Figure 3. Schematic diagram representing biocontrol efficacy assay for *R. solanacearum* antagonizing bacteria

F. Calculation of biocontrol efficacy

1. Calculate disease severity index (DSI) using the formula:

$$\text{DSI} = \left(\frac{\text{no. of diseased plants}}{\text{total number of plants inspected}}\right) \times 100\%$$

2. Calculate biocontrol efficacy (BE) using the formula:

$$\text{BE} = \left(\frac{(\text{DSI of } R.\ solanacearum \text{ treated control} - \text{DSI of antagonist treated group})}{\text{DSI of } R.\ solanacearum \text{ treated control}}\right) \times 100\%$$

3. Calculate the DSI in the groups, and BE of antagonizing bacterium from the records on 10th day post *R. solanacearum* inoculation.

Data analysis

1. Perform One-Way ANOVA on the calculated DSI and BE, with *P* value less than 0.05, between-treatments in order to test the performances of biocontrol bacteria in the treatment groups.
2. To further investigate the nature of the differences between the control and different treatment means, perform Duncan Multiple Range Test (DMRT) after a statistically significant ANOVA.
3. Perform descriptive statistics (mean and standard error) on the calculated DSI and BE across all the treatment groups.
4. Data (Figure 4) can be found in the original research article titled “Endophytes from *Gnetum gnemon* L. can protect seedlings against the infection of phytopathogenic bacterium *Ralstonia solanacearum* as well as promote plant growth in tomato”; doi:10.1016/j.micres.2020.126503, published in Microbiological Research (Agarwal *et al.*, 2020).

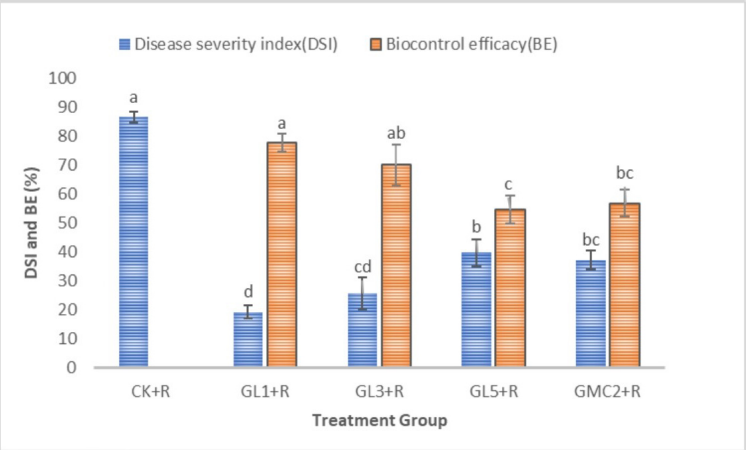

Figure 4. Biocontrol efficacy of bacterial isolates and respective disease severity index in treatment groups. CK+R: only *R. solanacearum* treated control, GL1+R: *Staphylococcus warneri* GL1+*R. solanacearum*, GL3+R: *Bacillus velezensis* GL3+*R. solanacearum*, GL5+R: *B.*

velezensis GL5+*R. solanacearum*, GMC2+R: *B. velezensis* GMC2+*R. solanacearum*. Different lower-case letters represent significant differences between treatments (one-way ANOVA, $P < 0.05$) according to Duncans multiple range tests. This figure is reprinted (with slight modification) from original research article Agarwal *et al.* (2020) with permission from Elsevier.

Notes

1. Confirm the antagonistic ability of bacterial isolate against *R. solanacearum* by any of the standard methods such as agar well diffusion, disc diffusion, or spot on lawn assay with at least three repetitions prior to start this *in planta* biocontrol efficacy assay.
2. Calculate the age of seedlings from the day of putting the seeds on the germination tray.
3. The culture media, and incubating conditions for optimal growth can be different for different *R. solanacearum* antagonizing bacteria. This change will not have any effect further, since the cell number is adjusted for all isolates and media contamination is removed from the bacterial cells after repeated wash with sterile water.
4. In the initial root bacterization stage, take some additional seedlings in order to compensate any damage/death of seedlings while handling.
5. The number of seedlings in treatment groups can be increased as per convenience and to ensure the biocontrol efficacy of bacterial isolates, repeat this assay at least three times.
6. This assay cannot be conducted for more than 10 days post *R. solanacearum* inoculation in tomato, as seedlings (control group-1, Table 1) are unable to sustain after this time period in sterile water without any supplements. The age up to which the seedlings can sustain in sterile water is a subject of consideration before trying this assay on other hosts.

Recipes

1. Casamino acid peptone glucose broth (pH 6.5-7.0) (100 ml)
   100 mg Acicase
   1 g Peptone
   500 mg Glucose
   Add distilled water up to 100 ml volume and autoclave at 121 °C under 15 psi pressure for 20 min

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Competing interests

The authors declare no conflict of interests.

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